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DEVELOPMENT OF A MODEL SYSTEM TO STUDY NK CELL HYPORESPONSIVENESS

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“**Llegar a la meta no es vencer,**
lo importante es el camino y en él,
caer, levantarse, insistir y aprender.”

Mägo de Oz - La Posada de los Muertos

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Abstract
Resumen

Natural Killer (NK) cells are cytotoxic lymphocytes important in immune responses to cancer and multiple pathogens because of their ability to kill pathogen infected and tumour cells, and to regulate the function of other immune cells by the secretion of soluble factors. It has been described that chronic activation of NK cells can induce a hyporesponsive state. This situation has been described as one evasion strategy used by tumours or viruses to escape immune system surveillance.

The molecular basis of the mechanisms that allows the generation and maintenance of this hyporesponsive state on NK cells are unknown, and the main objective of this thesis was to establish and in vitro system to induce hyporesponsiveness on healthy human NK cells to study the molecular basis of NK cell hyporesponsiveness.

IL-2 activated human NK cells treated with ionomycin lose their ability to degranulate when cocultured with different target cells, and to secrete IFN-γ even after IL-12 and IL-18 stimulation. However, they are able to degranulate when stimulated through CD16. Cytotoxic and secretory functions were restored by IL-2 stimulation. The expression of activating and inhibitory receptors was normal in ionomycin treated NK cells, however, we observed lower expression of the αL, αM and β2 integrins. Study of different NK killing steps demonstrated that ionomycin treated NK cells are less able to form conjugates with target cells. Transcriptomic analysis of activated NK cells treated with ionomycin revealed differential expression of multiple genes; however this transcriptional signature was different to those previously defined for other hyporesponsive lymphocytes. Treatment of freshly isolated NK cells from healthy donors with ionomycin induces a hyporesponsive state similar to that observed after the treatment of activated NK cells. However, these cells did not recover their ability to degranulate and produce IFN-γ entirely in the presence of IL-2, and are unable to exert degranulation as efficiently as control cells when stimulated through CD16.

Ionomycin treatment has shown to be an useful tool to understand the NK cell unresponsiveness induced after chronic activaton. NK cells. The molecular basis of the lack of response has not been fully elucidated. However the integration of the data described in this thesis provides a conceptual framework that will be useful to understand NK cell biology and NK cell hyporesponsiveness.

Las células "Natural Killer" (NK), son linfocitos citotóxicos capaces de inducir la lisis de células tumorales o infectadas por diferentes patógenos. También pueden regular la función de otras células inmunes mediante la secreción de distintos factores solubles. Sin embargo, una activación crónica de las células NK, puede dar lugar a un descenso de su capacidad efectora. Este fenómeno ha sido descrito como una posible estrategia usada por tumores o diferentes virus para escapar de la vigilancia mediada por el sistema inmune.

Actualmente se desconocen las bases moleculares de esta pérdida de actividad mantenida en las células NK, así, el objetivo principal de este trabajo fue establecer un sistema experimental para poder inducir y estudiar en células NK humanas de donantes sanos las bases moleculares de esta pérdida de función.

El tratamiento de las células NK humanas activadas con IL-2 con ionomicina provoca una pérdida de su capacidad de ejecutar un ataque citotóxico frente a diferentes células diana, y la de secretar de IFN-γ incluso tras su estimulación con las citoquinas IL-12 e IL-18. Sin embargo, si conservan capacidad de degranular cuando fueron estimuladas a través del receptor CD16. Las funciones citotóxica y secretora pudieron ser recuperadas en estas células cultivándolas en presencia de IL-2. Esta pérdida de función no fue debida a cambios en la expresión de receptores activadores o inhibidores, sin embargo, sí que se observó menor expresión de las integrinas αL, αM y β2. El estudio detallado de los distintos pasos del ataque citotóxico, mostró que las células NK desensibilizadas, eran incapaces de formar conjugados con células diana con la misma eficiencia que las células control. Análisis de la expresión génica de las células NK tratadas con ionomicina, permitió identificar diferencias en la expresión de múltiples genes, siendo este perfil génico distinto a los descritos en otras células inmunes que han perdido su funcionalidad. El tratamiento de células NK recién purificadas de donantes sanos con ionomicina, les induce un estado de pérdida de respuesta similar al observado en células activadas. No obstante, estas células no recuperan totalmente su capacidad de degranular y de secretar IFN-γ en presencia de IL-2; ni son capaces de responder a la estimulación por CD16 de manera tan eficiente como las células recién aisladas control.

El análisis de las células NK tratadas con ionomicina, muestra que esta estrategia puede ser un método eficaz para el estudio de la pérdida de función descrita en las células NK tras su estimulación crónica. Aunque más experimentos serán necesarios para lograr entender completamente este estado, los datos descritos en esta tesis pueden ayudar a comprender mejor la biología de las células NK y su pérdida de función.

Abbreviations

7-AAD	7-aminoactinomycin d
ADCC	Antobody dependant cell cytotoxicity
AICD	Activation induced cell death
AINR	Activation induced non responsiveness
ANOVA	Analysis of variance
APCs	Antigen presenting cells
APC	Allophycocyanin
BCR	B cell receptor
CD	Cluster of diferentiation
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
CMAC	7-amino-4-chloromethylcoumarin
cSMAC	Central supramolecular activation cluster
Ct	Cycle threshold
CTLA4	Cytotoxic T-lymphocyte antigen 4
DMEM	Dulbecco modified eagle medium
DMSO	Dimethyl sulfoxide
dNK	Decidual NK cells
DR4	Death receptor 4
DTT	Dithiothreitol
e.g.	Exempli gratia – For example
E:T	Effector : target
EGTA	Ethylene glycol tetra-acetic acid
ERM	Ezrin/radixin/moesin
EtOH	Ethanol
FACS	Fluorescent-activated cell sorter
F-actin	Filamentous actin
Fas-L	Fas-ligand
FBS	Fetal bovine serum
Fc	Antibody crystallizable region
FcγR	Fcγ receptor
FDR	False discovery rate
Fig.	Figure
FITC	Fluorescein isothiocyanate
fNK	Freshly isolated NK cells
GAM	Goat anti mouse
GAR	Goat anti rabbit
GM-CSF	Granulocyte macrophage colony stimulating factor
HBSS	Hank's balanced salt solution
HCST	Hematopoietic stem cell transplantation
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid

HIV	Human immunodeficiency virus
HLA	Human leukocyte antige
HPCs	Hematopoietic progentor cells
HRP	Horseradish peroxidase
HS	Human serum
ICAM	Intercellular adhesion molecule
IDO	Indoleamine-pyrrole 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
IL-12R	Interleukin 12 receptor
IL-18R	Interleukin 18 receptor
IL-18RAP	Interleukin 18 receptor adaptor molecule
IP_3	Inositol 1,4,5-trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
KIR	Killer-cell immunoglobulin-like receptor
Lamp1	Lysosomal-associated membrane protein 1
LFA-1	Lymphocyte function-associated antigen 1
mAbs	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
MCMV	Mouse cytomegalovirus
MDSCs	Myeloid-derived suppressor cells
MFI	Mean fluorescence intensity
MHC	Mayor histocompatibility complex
MIF	Macrophage migration inhibitory factor
min	Minutes
MOPC	Mouse igg1 kappa isotype antibody
mRNA	Messenger RNA
MTOC	Microtubule organizing center
n.s.	Non significative
NCAM	Neural cell adhesion molecule
NCR	Natural cytotoxicity receptor
NFAT	Nuclear factor of activated T-cells
NK	Natural Killer cell
NKT	Natural Killer T cell
p	P-value
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PD-1	Programmed death 1
PE	Phycoerythrin
PFA	Para-formaldehyde
PGE2	Prostaglandin E2
PI	Propidium iodide
PKC	Protein kinase C
PMA	Phorbol ester 12, 13-dibutyrate
pSMAC	Peripheral supramolecular activation cluster
PVDF	Polyvinylidene fluoride
qPCR	Quantitative PCR
RasGRP	RAS guanyl nucleotide-releasing protein
rhIL	Recombinant human interleukin
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RQ	Relative quantity
RT	Room temperature
RT-PCR	Real time-PCR
SD S	tandard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOCs	Store-operated Ca2+ channels
TBS	Tris buffer solution
TCR	T cell receptor
TGF-β	Transforming growth factor β
TILN	Tumour infliltrating lymph nodes
TLRs	Toll like receptors
TNF-a	Tumour necrosis factor α
TRAIL	TNF-related apoptosis-inducing ligand
U	Units
vs.	versus
WT	Wild type

Introduction

1. NATURAL KILLER CELLS

Organisms have evolved a powerful collection of defense mechanisms collectively known as the immune system to protect themselves against harmful environmental agents foreign to their body. The immune system must be competent to specifically recognize pathogens and provide effective protection, and at the same time, it must have the ability to self-regulate to minimize autoimmunity, and further to generate an immunological memory. First the innate immune response and later the adaptive response will ensure the eradication of infected or transformed cells, and the establishment of memory.

Natural killer cells (NK) are part of the immune system which were described for the first time in 1975 (Herberman et al. 1975; Kiessling et al. 1975) as large granular lymphocytes able to spontaneously kill certain target cells *in vitro* without any prior sensitization. They have been classified as part of the innate immune system due to their ability to mediate a rapid response on exposure to an infectious organism in the absence of prior antigenic stimulation, and for not being a response mediated by antigen-specific receptors. However, nowadays, it is also known that they share properties with adaptive responses such as clonal expansion, the generation of long-lasting immunologic memory, the need to be "educated" or "licensed", the requirement of priming for fully responsiveness, or their ability to shape adaptive responses (Sun et al. 2009; Vivier et al. 2011).

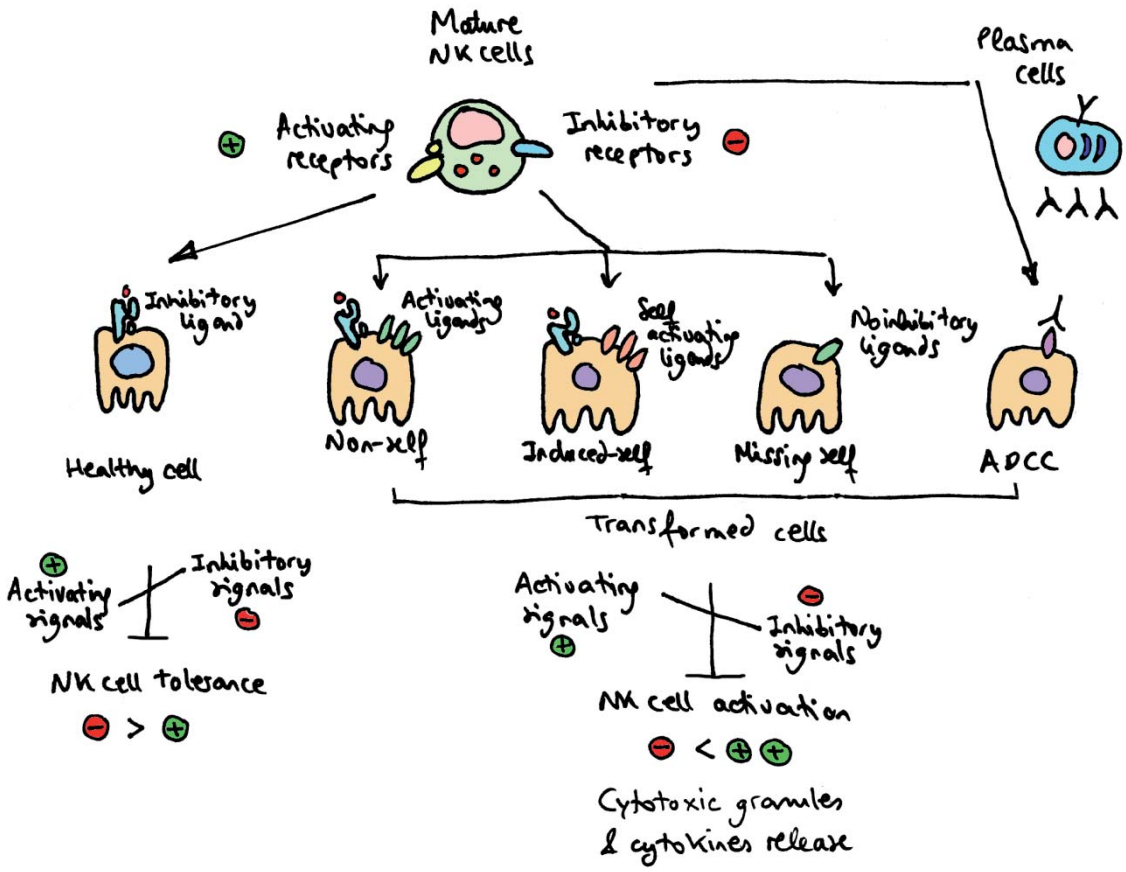
1.1 Origin and education of human NK cells

Natural killer cells are the third largest lymphoid cell population and represent between 10 and 15% of the lymphocytes in peripheral blood (Cooper et al. 2001). They share a $CD34^+$ hematopoietic progenitor with T and B lymphocytes (Miller et al. 1994; Galy et al. 1995) however; some studies report that NK cells can also be generated from myeloid progenitors (Grzywacz et al. 2010). NK cell development starts in the fetal liver, but after birth it occurs mainly in the bone marrow (Haller et al. 1977), although lymph nodes, the thymus, the gut and the liver are also sites of NK cell development (Freud et al. 2005; Di Santo and Vosshenrich 2006; Chinen et al. 2007; Wu et al. 2012). NK cell maturation requires extrinsic regulation by other immune cells as dendritic cells, monocytes and neutrophils, which provide maturation and survival signals that sustain their differentiation towards a mature NK cell (Soderquest et al. 2011; Ganal et al. 2012; Jaeger et al. 2012).

The different stages of NK cell lineage commitment, maturation and function are regulated by many transcription factors including E4BP4, ID2, BLIMP1, Eomes, ETS-1, HELIOS, KLF4, T-bet, or TOX (Ramirez and Kee 2010; Hesslein and Lanier 2011; Luevano et al. 2012; Cichocki et al. 2013; Huntington et al. 2013), and progress of the maturing NK cell through the different developmental stages has been correlated with the sequential acquisition of different cell surface receptor proteins such as c-kit (CD117), CD56, CD16, CD11b, CD25, CD57, CD94 or CD62-L (Huntington et al. 2007).

NK cells display a **heterogeneous array of cell surface receptors**, and signals from these receptors allow them to maintain self-tolerance and differentiate between normal cells and stressed/transformed or infected cells. NK cells determine if a cell should be considered a target or not, depending on the balance of signals originated from the many different activating and inhibitory receptors expressed by these cells (Vivier et al. 2004). For example if a putative target cell expresses low levels of self-major histocompatibility complex (MHC) class I molecules, then there will be a dominance of activating signals over inhibition; similarly, if there is an increased expression of activating ligands, but not inhibitory ligands, then the activation threshold will also be exceeded leading to activation (Raulet and Vance 2006) **(**Scheme 1**)**. Given that some of the ligands of activating receptors are self–molecules which normally are expressed at low levels (Long and Rajagopalan 2002), this **fine tuning** implies that NK cells have to undergo an education process that selects for those cells able to distinguish between healthy and stressed cells, allowing them to adapt their effector capacity to their host (Shafi et al. 2011). During this education process NK cells acquire receptor expression, the self-tolerance property, but also their effector function license. To date, there is still no consensus about how NK cells acquire their education, and different theories are still being discussed (Raulet and Vance 2006). However, all models proposed for NK cell licensing agree on an important role for the interaction between MHC class I molecules and the inhibitory receptors on NK cells during this process (Hoglund and Brodin 2010).

Most human NK cell clones express at least one inhibitory receptor which is acquired stochastically, and which recognizes self-MHC class I proteins (Valiante et al. 1997), but around 13% +/- 6% of circulating NK cells in an MHC sufficient host don't express any inhibitory receptor able to interact with self MHC class I (Anfossi et al. 2006). These cells, instead of being eliminated like thymocytes that cannot engage their TCRs with self-MHC class I molecules, are maintained, but they exhibit weaker cytolytic and cytokine production abilities after *in vitro* stimulation through several activating receptors (Fernandez et al. 2005; Kim et al. 2005; Anfossi et al. 2006; Yu et al. 2007). However, these hyporesponsive NK cells are able to respond robustly in case of viral (Tay et al. 1995; Polic et al. 1996; Sun and Lanier 2008; Orr et al. 2010), bacterial (Fernandez et al. 2005), protozoal infection (Denkers et al. 1993), PMA-Ionomycin stimulation (Fernandez et al. 2005), IL-2 culture (Johansson et al. 1997), IL-12 plus IL-18 stimulation (Yokoyama and Kim 2006), or transplantation (Yu et al. 2009).



Scheme 1: NK cell activation. NK cells express different activating and inhibitory receptors, which allow them to distinguish between healthy cells, which express inhibitory ligands; and transformed cells, which show an altered expression of activating or inhibitory ligands. The balance between the signals coming from activating and inhibitory receptors will determine if the NK cell becomes activated or not. Different scenarios can activate NK cells: appearance of non-self activating ligands, increased expression of self activated ligands, decreased expression of inhibitory ligands or opsonized target cells.

Circulating hyporesponsive NK cells are also found in MHC deficient human patients, although the recovery of their functionality after stimulation with cytokines is controversial (Zimmer et al. 1998; Furukawa et al. 1999; Vitale et al. 2002). Interestingly, both educated and hyporesponsive NK cells in mice express a mature phenotype in the periphery (Fernandez et al. 2005), indicating that their education most likely occurs after the achievement of a mature stage. In humans the hyporesponsive cells are also similar to normal NK cells in cell-surface phenotype, perforin and granzyme content, and ability to respond to PMA-Ionomycin, however lower levels of inhibitory receptors, DNAM1 and a reduced size of the $CD56^{bright}$ subset has been reported (Vitale et al. 2002; Anfossi et al. 2006). NK cells that develop in an MHC class I deficient host do not exhibit autoreactivity (Zimmer et al. 1998; Furukawa et al. 1999; Vitale et al. 2002), although recurrent bacterial infections and chronic inflammation have been described in TAP2 (Antigen peptide transporter 2) deficient patients (Vitale et al. 2002). Thus, MHC class I expression in the environment regulates NK cell function and those cells that don't fulfill the requirement for expression of an inhibitory receptor for self MHC class I are allowed to seed the periphery, but calibrated to be not functionally competent (unlicensed),

although able to respond under certain circumstances. Infection and inflammation have been shown to stimulate licensed and unlicensed NK cells in a similar way, and unlicensed NK cells can respond even more strongly, as shown in MCMV infection (Orr et al. 2010) (although this activity is controversial (Sungur et al. 2013)), and in ADCC. Indeed, in this context unlicensed NK cells could react better against tumour cells precisely because they are not inhibited by self-MHC class I, suggesting a novel therapeutic approach for use in neuroblastoma (Tarek et al. 2012) and demonstrating the potential functionality of unlicensed NK cells when the restriction to respond is overridden.

NK cell responsiveness is not an on/off situation, instead it is thought to be gradual, as NK cells that express more inhibitory receptors for self MHC class I, are also more responsive (Yu et al. 2007; Brodin et al. 2009; Joncker et al. 2009), and hosts that express different MHC class alleles also generate greater responses (Johansson et al. 2005). This implies that each NK cell is going to undergo a **tuning process depending on the number and type of inhibitory receptors, the strength of the engagement, the stimulatory receptors expressed, and the ligands expressed by the host, establishing a threshold or set point, and the signaling necessary for response is going to vary depending on the cell** (Joncker et al. 2009). Importantly, the engagement of only one inhibitory ligand for each NK cell clone, despite the expression of multiple different inhibitory receptors, is sufficient to inhibit the NK cell response (Yu et al. 2007).

This threshold is not fixed during development, and **mature NK cells can also 'tune' their capability to react**. This was first demonstrated by *Johansson* et al, in mice with mosaic expression of MHC class I (Johansson et al. 1997), and also in experiments where mature WT NK cells were transferred into mice constitutively expressing an activating ligand (Tripathy et al. 2008). Later this plasticity or recalibrating ability has been further demonstrated. When NK cells generated in MHC class I deficient mice are placed in an MHC class I-sufficient setting, they are able to readapt and don't cause autoimmunity to their new host, and in the same manner, functional mature NK cells lose their effector capabilities when placed in an environment devoid of MHC class I molecules (Elliott et al. 2010; Joncker et al. 2010).

Further support for this model of constant education and tuning of NK cells, comes from studies in human leukemia where KIR mismatched hematopoietic stem cell transplantation (HSCT) is used. Normally HSCT consist of a graft depleted in T and B cells, where mature NK cells and hematopoietic progenitors cells are transferred into the patient. During the first months after transplant, the presence of mismatched NK cells expressing KIRs not adapted for the donor favours the elimination of host leukemic cells, host APCs and exacerbated T cells that could cause *graft versus host disease*. Indeed, not only licensed NK cells are alloreactive in this setting, but non-licensed NK cells also become reactivated (Hsu et al. 2005; Yu et al. 2009). However, this NK cell alloreactivity disappears approximately 3 months after transplant, when the circulating NK cell pool begins to represent mainly reconstituted cells, and the new NK cells, generated from the $CD34^+$ progenitors, became tolerant, not for the HLA they express, but for the HLA of their new host (Ruggeri et al. 1999; Ruggeri et al. 2002; Babor et al. 2013).

KIR/MHC class I interactions are not the only factors able to shape NK cell response. Constitutive expression of NKG2D ligands (Oppenheim et al. 2005; Wiemann et al. 2005) or m157 (Sun and Lanier 2008; Tripathy et al. 2008; Mazumdar et al. 2013), an MCMV encoded ligand for the murine Ly49H activating receptor, renders NK cells from these mice hypofunctional for stimulation via these, and other, receptors (Sun and Lanier 2008; Tripathy et al. 2008), insensitive to IL-12 plus IL-18 stimulation (Tripathy et al. 2008) and reduces either the number of receptor positive cells (Sun and Lanier 2008; Tripathy et al. 2008) or induces a downregulation of the receptor (Oppenheim et al. 2005; Wiemann et al. 2005). In the m157 model it was shown that this hyporesponsiveness was reversible in the WT NK cells when they were stimulated with IL-2, but not in m157 expressing NK cells because of continuous *cis* interaction (Tripathy et al. 2008), although both WT and m157 expressing NK cells were functional when stimulated with PMA/Ionomcycin (Tripathy et al. 2008). In the case of transgenic mice constitutively expressing NKG2D ligands, the higher complexity of the system due to the possible role for soluble ligands, and their effects on NK cells and CD8+ T cells expressing NKG2D, weakens the conclusions. However in experiments done *in vitro* where NK cells are cultured with targets expressing NKG2D ligands for three days, the interaction is able to shut down NK cell responses mediated not only by NKG2D but also via other receptors (Coudert et al. 2005; Coudert et al. 2008). This role of activating receptors in education has also been shown in humans where NK cells expressing the activating receptor KIR2DS1 are inefficient if derived from donors bearing its ligand HLA-C2 (Fauriat et al. 2010). In agreement with these observations it has also been described that mice deficient in either the activating receptor NKp46 (Narni-Mancinelli et al. 2012), or in NKG2D (Zafirova et al. 2009) generate hyperactivated NK cells, demonstrating that activating receptors interaction with their endogenous ligands, is also associated with downmodulation of NK cell responsiveness. Overall these data imply that **activating ligands** expressed in the bone marrow **also collaborate in NK cell education**, shaping their responsiveness to their host, and that these cells able to recognize the ligand, instead of being deleted, analogous to the elimination of thymocytes that have high affinity for self-peptide-MHC complexes, are rather maintained, but in a hyporesponsive state.

This education process confers to NK cells the properties of being tolerant to self and reactive to non-self, but also ensures the plasticity of their responsiveness thanks to a dynamic evaluation of the environment that occurs during the entire lifetime of the NK cell. The mechanisms involved are not well understood, but education and differentiation are uncoupled processes, since uneducated NK cells are able to differentiate and proliferate, maintaining their cytotoxic potential under control (Bjorkstrom et al. 2010). These results also suggest that NK cells may end-up deregulated and in a hyporesponsive state after excessive exposure to their ligands, which could be overcome if the pro-inflammatory environment persists.

1.2 Functions of NK cells

NK cell are involved in both innate and adaptive immune responses. They are able to participate in an early response as they can directly eliminate virally infected or transformed cells through target-cell lysis, but they can also exert a range of other functions including the recruitment of other innate immune cells such as neutrophils and macrophages, the activation or elimination of dendritic cells, and the priming of adaptive lymphocytes (T or B cells) through the release of soluble factors. Thus NK cells can act to integrate innate and adaptive responses that will act in concert. They also have important roles in pregnancy (Moffett et al. 2004) immune homeostasis (Flodstrom-Tullberg et al. 2009), and can display long lasting memory (Vivier et al. 2008).

There is increasing evidence that this versatility is possibly due to the existence of distinct NK-cell subsets. These may represent different lineages of NK cells, for example uterine NK cells represent a very specialized subset of NK cells which express a unique set of receptors and functions (Moffett-King 2002). Also, other populations as liver NK cells which appeared to originate from hepatic hematopoietic progenitor cells (HPCs) but not from the bone marrow (Peng et al. 2013); and a subpopulation of IL-22 producing NK cells dependant on the RORγT transcription factor, instead of E4BP4, and which are found mainly in mucosal tissues has also been described (Cella et al. 2009). Classically NK cells have been divided between $CD56^{dim}$ - $CD16^{bright}$ cells, which are mainly cytotoxic cells and respond better to target-cell expressed ligands, and $CD56^{bright}$ - $CD16^{-}$/$CD16^{dim}$ cells which are potent secretory cells and respond more quickly to IL-12 or IL-15 triggering (Jacobs et al. 2001). A subset of $CD56^{-}CD16^{+}$ cells is also found in blood of healthy individuals. These $CD56^{-}$ cells possess lower killing and cytokine production abilities than $CD56^{+}$ cells, and were found expanded in chronic HIV and hepatitis C infection, following allogenic hematopoietic transplantation and in cord blood (Bjorkstrom et al. 2010). The majority of NK cells in circulation (90%) are $CD56^{dim}$ cells, while in secondary lymph tissues the NK cell population is primarily $CD56^{bright}$ (Vosshenrich et al. 2006).

To exert their functions, mature resting NK cells need priming from cytokines (Trinchieri 1995; Chaix et al. 2008) or other immune cells (Ganal et al. 2012), but tumor cells (North et al. 2007) or exosomes (Reiners et al. 2013) have also been proposed to be able to provide these signals.

Natural killer cells carry out their functions through two major effector mechanisms. The first described, and the one for which they are named, is their **cytotoxic role**. However, NK cells are also **potent regulators of innate and adaptive immune system responses due to their ability to secrete a range of different cytokines and chemokines**.

Among the cytokines produced by NK cells can be found pro-**inflammatory cytokines** such as IFN-γ, TNF-α or IL-6; **immunosuppressive cytokines** including IL-10, IL-13 or TGF-ß, **growth factors** such as GM-CSF, G-CSF, IL-3; and a range of different **chemokines** including XCL1, CCL1, CCL2, CCL3, CCL4, CCL5, CCL8, CCL17, CCL26, CXCL8, CXCL16, XCL1 or CXCL8 (Souza-Fonseca-Guimaraes et al. 2012). Resting NK cells contain preformed IFN-γ mRNA (De Maria et al. 2010), and are an important early source of this inflammatory cytokine. IFN-γ production by NK cells is known to shape the Th1 immune response, activate APCs to upregulate expression of MHC class I and class II molecules, activate macrophage killing of intracellular pathogens, and have antiproliferative effects on viral and malignant transformed cells (Schroder et al. 2004; Strowig et al. 2008). Natural killer cells can also secrete other immune mediators as nitric oxide (NO), indoleamine 2,3-dioxygenase (IDO) or α-defensins (Souza-Fonseca-Guimaraes et al. 2012).

NK cell cytotoxicity, and the induction of target cell apoptosis, depends on two different pathways: the **granule exocytosis** pathway and the death receptor pathway. Experiments done at the single cell level have shown that most mature NK cells are unable to directly exert 'natural' cytotoxicity in isolation, highlighting their need to be primed for efficient killing (Guldevall et al. 2010; Christakou et al. 2013). Resting NK cells contain preformed specialized secretory lysosomes that upon target cell recognition are polarized and rapidly exocytosed, so that they secrete their content into the site of contact between the NK cell and the target cell, the immune synapse. The components of these cytotoxic granules include the proteases granzymes (A, K, B, H, M), pore forming protein perforin, Fas ligand (FasL), TRAIL, granulysin, cathepsin, cathelicidin and defensins 1-3 (Krzewski and Coligan 2012) and cause caspase dependent and independent apoptosis of the target cell.

The **death receptor pathway** involves the secretion of FasL, TNF-α or TRAIL. Similar to the granule exocytosis method, upon stimulation and synapse formation these ligands are polarized and secreted resulting in a caspase-dependent apoptosis of the target cell (Vujanovic et al. 1996; Wallin et al. 2003; Smyth et al. 2005). NK cells are also able to augment the expression of death receptors on target cells through the release of IFN-γ (Siegmund et al. 2005).

These apoptosis mechanisms will ensure that in the case of viral infection, the viral load will be destroyed together with the target cell.

1.3 NK cell receptors

T or B cells populations express a diverse set of antigen-specific receptors, however each clone only expresses a single antigen receptor. Unlike T and B lymphocytes, **each NK cell displays a heterogeneous array of activating and inhibitory cell surface receptors**, which **cooperatively** makes them able to discriminate between normal or unhealthy cells depending on the balance of signals induced by them (Vivier et al. 2004). Some of these receptors are expressed in a stochastic pattern, resulting in many subsets of functionally distinct NK cells.

NK express many different **activating receptors** that allow them to recognize target cells through different strategies. With the exception of the CD16 receptor which is able to act alone, they act as **coactivating receptors** as they need to cooperate in order to elicit a response (Augugliaro et al. 2003; Bryceson et al. 2006). This need for synergistic activation, could serve as a mechanism of control for NK cells or as a way to detect and react to different targets and elicit different types of responses. Many of the activating NK cell receptors recognize self-antigens that under normal situations are not expressed or expressed at low levels, as for example the NKG2D receptor which recognize MIC and ULBP molecules which are encoded in human genome, and can be upregulated after viral infection, genotoxic damage or tumor transformation, a strategy called *stress induced self recognition* (Mistry and O'Callaghan 2007) **(**Scheme 1**)**. Other NK cell receptors are also able to directly recognize *non self* molecules as the NKp46 receptor which can recognize viral haemaglutinin (Arnon et al. 2001; Mandelboim et al. 2001). The low-affinity Fc receptor CD16 (FCγRIII), allow NK cells to detect opsonized cells and mediate *antibody dependent cell cytotoxicity* (ADCC) (Ojo and Wigzell 1978) **(**Scheme. 1**)**. Most of these activating receptors lack intracellular signaling motifs. Instead, they associate noncovalently via a positively charged residue (lysine or arginine) in the transmembrane with homo or heterodimeric signal-transducing adaptor molecules, that contain an aspartate residue in the transmembrane, and either one immunoreceptor tyrosine-based activating motif (ITAM) in the cytosol (FcεRγI and DAP12), three ITAMs (CD3-ζ) (Wirthmueller et al. 1992), or the Tyr-Ile-Met-Asn motif of the adaptor DAP10 (Wu et al. 1999). Other molecules such as SAP can also act as signal-transducing adaptors for NK cell activation (Meinke and Watzl 2013). After the engagement of the receptor, Src family protein tyrosine kinases became activated and phosphorylate the tyrosine residues of the ITAMs. This phosphorylation recruits and activate Syk or ZAP70 tyrosine kinases in the case of ITAM adaptors, or PI3-kinase and Grb2-Vav1 in the case of DAP10 (Lanier 2008).

Most of the **inhibitory receptors** expressed by NK cells recognize **self MHC class I molecules:** the NKG2A/CD94 heterodimer interacts specifically with non classical HLA-E (Lee et al. 1998) while the Killer-cell immunoglobulin-like receptors (KIRs) are specific for classical MHC class Ia ligands HLA-A, B and C (Wagtmann et al. 1995). The LILR genes ILT2 and ILT4 can bind both classical and non-classical HLA molecules including HLA-G, and –F (Navarro et al. 1999; Lepin et al. 2000). Thus loss of MHC Class I expression can lead to NK cell activation via loss of inhibition by MHC class I expression: the "missing-self" hypothesis **(**Scheme. 1**)**. In this way NK cell activation by loss of MHC Class I is a complementary way of activation to that used by $CD8^+$ T cells where the TCR positively recognizes MHC class I molecules, acting as a synergic strategies for target cell recognition (Karre et al. 1986). The expression of KIR and CD94 proteins occurs in a stochastic manner, and each NK cell expresses its own repertoire of receptors (Valiante et al. 1997; Raulet et al. 2001). Further, since multiple alleles of KIR genes are present in the population and the KIR locus also varies in the number of genes present (Suto et al. 1998; Parham 2005), a **variegated diversity of inhibitory receptor expression is generated, with distinct patterns of reactivity for each host**. NK cells also express **non-MHC inhibitory receptors** such as KLRG1, TIGIT, Siglecs, LAIR-1, or CEACAM1 that bind a diverse set of ligands including cadherins, sialic acid or collagen. All the inhibitory

receptors in humans contain intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIM) in their cytoplasmic tail. Following inhibitory receptor interaction with its ligand and crosslinking, the phosphorylation of tyrosine residues within the ITIM elements by Src family protein tyrosine kinases recruited to the synapse by activating receptors (Blery et al. 1997), lead to the recruitment of cytoplasmic tyrosine phosphatases 'SH2 domain-containing protein tyrosine phosphatase' 1, or2 (SHP-1 or SHP-2) (Vely et al. 1997) for KIR or CD94/NKG2A receptors, or the lipid phosphatase 'SH2 domain-containing inositol phosphatase' (SHIP) which acts as a 5'inositol phosphatase, in the case of 2B4, KLRG1 or TIGIT (Tessmer et al. 2007; Liu et al. 2012). Several signaling molecules involved in NK cell activation can be targets for SHP mediated dephosphorylation including Src kinases, ITAMs, Zap70, Syk, PLCγ, Vav, LAT, PI3'kinase or SLP76. In parallel to this dephosphorylation, ITIM-containing receptors also mediate phosphorylation events as the disruption of the signaling complex c-Cbl with CRK and C3G, by phosphorylation by c-Abl (Peterson and Long 2008). Inhibition occurs very early after NK cell-target cell contact, preventing the generation and propagation of activating signals, rather than silencing them (Abeyweera et al. 2011).

Integrins, immunoglobulin superfamily receptors, selectins, cadherins and mucins comprise the five major groups of cell **adhesion molecules**. Integrins are cell-surface transmembrane glycoproteins that are expressed as α-ß heterodimers and mediate multiple cell-cell and cell-matrix interactions. They are involved in survival/apoptosis, proliferation, cell-cycle progression, cell shape, polarity, adhesion, migration and differentiation. Integrins exist in different conformational states. Newly synthesized integrin arrives to cell surface in a "bent" or closed conformation. Upon activation by "inside-out signals" they change to an open conformation and form clusters (Luo et al. 2007). Finally, upon ligand binding, "outside-in signals" will lead to the final activated conformation. To transmit their signals, ß2 chain integrins associate with different molecules after phosphorylation of the ß2 chain or its cleavage by Cathepsin X (Hogg et al. 2011; Jevnikar et al. 2011). The importance of the ß2 chain in NK cell maturation and function, migration (Crozat et al. 2011), and lytic granule polarization has been described; being the signalling through LFA-1 (αMß2 integrin) sufficient to induce granule polarization, but not degranulation (Bryceson et al. 2010), and stimulation with a ß2 chain activating antibody is enough to increase killing (Sadhu et al. 2007). Integrins expressed by NK cells include LFA-1, Mac-1, LFA-3, VLA-4, VLA-5. Other adhesion molecules expressed by NK cells are PSGL-1, CD2, DNAM1, CD96, CD44, ICAM-1, L-selectin (mainly expressed by $CD56^{bright}$) and PEN5 (mainly expressed by $CD56^{dim}$) (Farag and Caligiuri 2006).

Natural killer cells express constitutively **interleukin receptors**. The $CD56^{bright}$ subset express the high affinity IL-2 receptor together with c-kit, which enhances IL-2 induced proliferation. The $CD56^{dim}$ NK cells, in contrast, express only the low affinity IL-2 receptor, and are $c\text{-}kit^{-}$. NK cells also express receptors for IL-1, IL-10, IL-12, IL-15, IL-18, and in general higher levels of these receptors are found expressed by the $CD56^{bright}$ subset. Different **chemokine receptors** are also found in NK cells and these again are differentially expressed between the $CD56^{bright}$ and $CD56^{dim}$ subsets (Inngjerdingen et al. 2001).

The differential expression of all these receptors between $CD56^{dim}$ and $CD56^{bright}$ subpopulations influences how they respond to different stimuli, and their homing to lymphoid tissues and sites of inflammation (Cooper et al. 2001).

The importance of the nanoscale organization of NK cell receptors was highlighted using superresolution microscopy in murine (Guia et al. 2011) and human (Pageon et al. 2013) NK cells. By analyzing the transit time of activating and inhibitory surface receptors through different volumes of educated and uneducated NK cells, *Guia* et al. demonstrated that NK cell receptors were differentially restricted in their movement at the cell surface. Activating receptors were confined in nanodomain structures favorable for signaling, while inhibitory receptors were trapped by the actin meshwork structures. However, when these experiments were repeated in uneducated NK cells, taken from mice deficient in MHC class I, activating and inhibitory receptors were both confined together by the actin meshwork. In complementary experiments *Pageon* et al., studied the organization of inhibitory receptors, and how activation of the NK cell through NKG2D influences the clustering of KIR2DL1 mediated by actin cytoskeleton. These articles demonstrate the existence of **crosstalk between activating and inhibitory receptors on their membrane localization** and suggest a mechanism for signal integration and control of NK cell activation.

1.4 The NK cell killing process

The killing of target cells by NK cells is a rapid, multi-step process that occurs in sequential stages beginning with initial adhesion and continuing all the way to the directed release, or not, of cytolytic granules (Orange et al. 2003). Normally, NK cell activation *in vivo* depends on the coordinated action of various receptors with activating potential (Bryceson et al. 2006; Kim et al. 2010), although signalling through CD16 alone can result in granule release if sufficient stimulation is given, while stimulation of LFA-1 by itself can induce polarization (Bryceson et al. 2005). Resting NK cells contain preformed lytic granules, so the entire cytolytic process must be very tightly regulated at the different check points during the killing steps.

In vitro experiments have shown that NK cells can form an effective cytolytic synapse against one target cell, while simultaneously receiving inhibitory stimulation from other sites (Eriksson et al. 1999) so signals from inhibitory and activating synapses are spatially restricted to one area.

Following the first contact that is mainly stabilized by the interaction of L-selectins, integrins and CD2 with the susceptible target cell, when the integration of signals from activating and inhibitory receptors results positive, different molecular reorganization steps take place, which will allow the formation of an immune synapse. The NK cell stops and spreads, and LFA-1 and Mac-1 segregate forming a peripheral supramolecular activation cluster (pSMAC) creating a firm conjugate between

both cells, and generating initial activation signals. Subsequently other molecules such as talin, ezrin-radixin-moesin, and Wasp polarize to the pSMAC. All these signals induce actin polymerization and the accumulation of filamentous actin at the pSMAC. At the same time, activating receptors in lipid rafts platforms cluster and accumulate in the central supramolecular activation cluster (cSMAC) where their synergistic interactions potentiate signaling leading to the recruitment of signal transduction molecules. These signals from the pSMAC and the cSMAC synergize to contribute to polarization of the MTOC and lytic granules. The cSMAC also plays roles in receptor synergy, receptor degradation and signal termination (Cemerski et al. 2008).

Lytic granules move along microtubules towards the MTOC thanks to dynein-dynactin motors, and then translocate with the MTOC toward the immunological synapse. Once the MTOC polarizes to the site of contact it get anchored at the plasma membrane, and lytic granules have to travel through hypodense regions of the cortical actin network helped by the myosin IIA motor protein and kinesin, and the action of different docking and priming proteins. Finally lytic granules fuse with the plasma membrane of the immune synapse in a process mediated by proteins of the SNARE family. The fusion with the membrane will create a pore allowing the secretion of the granule content. Partial fusion has also been described causing minimal secretion. Not all of the lytic granules are secreted at once, allowing NK cells to carry out serial target cell killing, and this might be due to the lower penetrability of the cortical actin area that limits the number of granules that can be exocytosed (Rak et al. 2011). After the killing of the target cell, the NK cell detaches and the immunological synapse is disassembled.

If the balance of signals is negative, spreading and stop signals will not be generated, KIR receptors will cluster to the cSMAC, while adhesion molecules polarize to the pSMAC. With time inhibitory receptors disperse in the immunological synapse, while adhesion molecules polarize to the center. Finally both cells detach and the inhibitory immune synapse becomes disrganised. Formation of the inhibitory synapse is not associated with the accumulation of filamentous actin and lipid raft polarization.

Although these general characteristics are now well established (Krzewski and Strominger 2008; Orange 2008; Krzewski and Coligan 2012; Vanherberghen et al. 2013), many questions still remain unanswered about the details of the NK cell killing process, and for example, analyses at the single cell level showed a very high variability between different NK cells from the same donor (Vanherberghen et al. 2013).

2. Immune evasion strategies

The fact that immunocompetent individuals may develop cancer, or suffer infections, implies that the immune system does not always succeed in its mission, and while organisms evolve better defense strategies, pathogens and tumors are also able to generate new escape manieuvers in response to the selective pressure imposed by the immune system. Indeed, this escape from immunosurveillance has been proposed as another hallmark, or biological capability, acquired during the development of tumors (Hanahan and Weinberg 2011). Evasion mechanisms are very diverse, varying from passive to highly aggressive strategies and include dormancy, the use of immune-privileged regions, secretion of molecules to alter immune responses or alteration of antigen display. This anti-immune response can also affect directly the immune system causing immunosuppression and allowing the appearance of opportunistic diseases.

2.1 NK cell evasion

NK cell evasion mechanisms generated by virus and tumor cells can be divided in two groups: Those able to mask transformed or infected cells, and those that affect directly the NK cell and modulate its response. This diverse panel of mechanisms highlights the importance of NK cell function in driving protection against tumors and pathogens.

In the group of strategies that involve making the altered cells "invisible", changing their phenotype to avoid NK cell recognition, are found changes in the repertoire of ligands exposed by cell; the upregulation of classical MHC class I molecules or homologues (Beck and Barrell 1988; Classen et al. 2003; Balsamo et al. 2012) or the decrease of activating receptor ligands by shedding (Raffaghello et al. 2004; Kaiser et al. 2007; Ashiru et al. 2010; Reiners et al. 2013), sequestering them inside the cell (Dunn et al. 2003; Fuertes et al. 2008), targeting them for degradation (Nejentsev et al. 2008), by expressing different microRNAs (Krmpotic et al. 2002; Stern-Ginossar et al. 2008) or by cytokines secreted by tumor-infiltrating inflammatory cells (Mishra et al. 2013).

Those mechanisms that directly affect NK cell function include the secretion of activating receptor antagonists that will cause the loss of expression of the activating receptor (Groh et al. 2002), the blocking of Ca^{2+} channels that will block the activation signal (Zocchi et al. 1998), the secretion of chemokine or cytokine inhibitors (Inngjerdingen et al. 2001), the activation of T regulatory cells that will control NK cell proliferation and cytotoxicity (Ghiringhelli et al. 2005), tumor microenvironment hypoxia (Sarkar et al. 2013), or the secretion of soluble factors as IL-10 (D'Andrea et al. 1993), TGF-ß (Lee et al. 2004; Wilson et al. 2011), PGE2 (Pietra et al. 2012), MIF (Krockenberger et al. 2008; Reiners et al. 2013), IDO (Pietra et al. 2012), viral hemagglutinin (Mao et al. 2010), tumor derived mucins (Zhang et al. 1997; Patankar et al. 2005) or L-Kynurenine (Della Chiesa et al. 2006). It has also been proposed, that NK cells can be converted into immunosuppressive MDSCs by tumor-derived GM-CSF (Kang et al. 2013).

Apart from this immunoinhibitory tumor microenvironment, another evasion strategy that has

been proposed is the **induced hyporesponsiveness** of NK cells (Hoglund and Brodin 2010) or "split anergy" (Jewett and Tseng 2011). However, cells in split anergy have been described to show increased cytokine production (induced by CD16 and IL-2) (Jewett and Tseng 2011).

The first *in vitro* experiments of target induced NK cell hyporesponsiveness trying to understand the nature of unresponsive NK cells were performed by Brahmi *et al.* in 1985 (Brahmi et al. 1985), where they showed that after interaction with target cells during 4 hours, NK cells lost their lytic potential towards the same or other target cells. Moreover, they also concluded that this NK cell inactivation was independent of calcium, as inactivation can be induced also in the presence of EGTA supplemented with Mg^{2+}, although cell-cell contact and intracellular Ca^{2+} pools were necessary. This article, and the following ones published by Z.Brahmi *et al*, represents the first effort to study induced NK cell unresponsiveness. They described that cell-cell contact was necessary for inducing hyporesponsiveness, and it caused also lost of the NK cell ability to mediate ADCC (Abrams and Brahmi 1988). However, the ability of unresponsive NK cells to form conjugates was maintained. They also showed that NK cells partially recovered their cytolytic activity after treatmentwith IFN-ß, and completely on exposure to IL-2 (Abrams and Brahmi 1986). Unresponsive NK cells were not able to inhibit fresh NK cells (Xiao and Brahmi 1989). Phenotypically they only found an increase of CD11b expression (Abrams and Brahmi 1988) and biochemically they observed weak PKC translocation (Shenoy and Brahmi 1991) and no phosphatidylinositol turnover (Gibboney et al. 1992) in K562 induced unresponsive NK cells. Moreover, they also described an induced downregulation of perforin and granzyme mRNAs (Bajpai et al. 1991).

Since this work, many articles have described examples of NK cell hyporesponsiveness. These include, *in vitro* experiments where NK cells are incubated for prolonged times with target cells (Jewett and Bonavida 1995; Jewett and Bonavida 1996; Coudert et al. 2005; Oppenheim et al. 2005; Wiemann et al. 2005; Roda-Navarro et al. 2006; Balsamo et al. 2009; Hasenkamp et al. 2010; Johann et al. 2010; Gill et al. 2012), transgenic mouse models where there is continuous expression of ligands for NK cell activating receptors (Oppenheim et al. 2005; Tripathy et al. 2008; Bolanos and Tripathy 2011; Mazumdar et al. 2013), in NK cells isolated from patients with different types of cancer (Hersey et al. 1979; Neri et al. 1981; Tursz et al. 1982; White et al. 1982; LeFever and Funahashi 1991; Espi et al. 1996; Konjevic et al. 1999; Costello et al. 2002; Doubrovina et al. 2003; Schleypen et al. 2006; Ciszak et al. 2007; Konjevic et al. 2007; Carrega et al. 2008; Verhoeven et al. 2008; Conry et al. 2009; Garcia-Iglesias et al. 2009; Szczepanski et al. 2009; Piroozmand and Hassan 2010; Al Omar et al. 2011; Mamessier et al. 2011; Platonova et al. 2011; Balsamo et al. 2012; Pietra et al. 2012; Fregni et al. 2013; Reiners et al. 2013) or viral infections (Confer et al. 1990; Sirianni et al. 2002; De Maria et al. 2003; Mavilio et al. 2003; Mao et al. 2010; Murugin et al. 2011; Yoon et al. 2011; Dupuy et al. 2012) or mouse models of cancer (Gill et al. 2012), it has been observed that these NK cells when tested *ex vivo* or *in vivo*, and exposed to another target cell, or another unrelated recognition strategy (ADCC or missing self recognition for example), are unable to efficiently exert their functions.

However, despite all these examples, very little is known about the molecular events that cause this loss of NK cell function.

In some of these studies NK cell function could be recovered by treatment with poly (I:C) which activates NK cells by engaging Toll-like receptor 3 *in vivo* (Oppenheim et al. 2005), but not in others (Tripathy et al. 2008). Similarly treatment with IL-2 was reported to be able to totally restore NK cell function in some articles (Abrams and Brahmi 1986; LeFever and Funahashi 1991; Coudert et al. 2005; Konjevic et al. 2007; Tripathy et al. 2008; Reiners et al. 2013), but not in others (Jewett and Bonavida 1995; Jewett and Bonavida 1996; Fregni et al. 2013). Treatment with other stimuli including IL-12 (Mazumdar et al. 2013), IL-15 (Verhoeven et al. 2008; Szczepanski et al. 2009; Balsamo et al. 2012), IFN-ß (Abrams and Brahmi 1986; Mazumdar et al. 2013), IFN-α (Konjevic et al. 2007), and TNF-α have also been reported to a produce partial restoration of NK function (Mazumdar et al. 2013). Intriguingly, the function of these hyporesponsive NK cells was also recovered after viral infections (Mazumdar et al. 2013).

In some experiments the hyporesponsive NK cells could degranulate when exposed to P815 cells loaded with CD16 antibody (De Maria et al. 2003; Mavilio et al. 2003; Fauriat et al. 2007), and stimulating the activity of CD16 has been demonstrated both *in vitro* and *in vivo* to be a promising strategy able to overcome NK cell inhibition and increase tumor cell killing by using bispecific antibodies which targets CD30 on tumor cells and CD16 on NK cells (Reiners et al. 2013). However, in other studies, ADCC activity was also lost (Brahmi et al. 1985).

Some articles also reported an altered phenotype of hyporesponsive cells; $CD16^{dim}CD2^{dim}CD56^{dim}CD69^{bright}CD11b^{bright}$ (Jewett and Bonavida 1995; Jewett and Bonavida 1996), NCR^{dull} (Sivori et al. 1999; Costello et al. 2002; De Maria et al. 2003; Mavilio et al. 2003; Fauriat et al. 2007; Garcia-Iglesias et al. 2009; Szczepanski et al. 2009; Johann et al. 2010; Mamessier et al. 2011; Platonova et al. 2011; Sanchez-Correa et al. 2011; Dupuy et al. 2012; Pietra et al. 2012). Lower levels of DNAM1 (Mamessier et al. 2011; Mamessier et al. 2011; Sanchez-Correa et al. 2011), 2B4 (Fauriat et al. 2006; Mamessier et al. 2011), NKG2D (Konjevic et al. 2007; Garcia-Iglesias et al. 2009; Szczepanski et al. 2009; Mamessier et al. 2011; Mamessier et al. 2011; Sanchez-Correa et al. 2011; Gill et al. 2012; Pietra et al. 2012; Reiners et al. 2013), CD94/NKG2C (Sanchez-Correa et al. 2011), CD16 (LeFever and Funahashi 1991; Lai et al. 1996; Patankar et al. 2005; Fauriat et al. 2006; Schleypen et al. 2006; Mamessier et al. 2011; Platonova et al. 2011), NKp80 (Mamessier et al. 2011; Platonova et al. 2011), NKp46 (Al Omar et al. 2011; Gill et al. 2012), NKp30 (Mamessier et al. 2011), NKp46 (Fregni et al. 2013), ILT2 (Platonova et al. 2011), CD6 (Sanchez-Correa et al. 2011), CD161 (Konjevic et al. 2007; Dupuy et al. 2012), CD56 ($CD56^{dim/-}$) (Mavilio et al. 2003; Schleypen et al. 2006; Sconocchia et al. 2009; Johann et al. 2010), CD57 (Bruno et al. 2013), CD49b (Gill et al. 2012), CD57, KIR and TRAIL (Mamessier et al. 2011), granzyme A (Schleypen et al. 2006; Pietra et al. 2012), perforin (Carrega et al. 2008; Al Omar et al. 2011), perforin and granzyme B (Schleypen et al. 2006; Mamessier et al. 2011) have also been reported to be characteristics of hyporesponsive NK cells. Upregulation of NKG2A, CD85j, CD27 or CD94 (Mamessier et al. 2011), the $CD56^{bright}$ $CD16^{-}$ population (Carrega et al. 2008), or the appearance of PD1 (Benson et al. 2010), CD27 and CD70 (Titanji et al. 2008), KIRs (Mavilio et al. 2003; Al Omar et al. 2011), LIR1 (Mavilio et al. 2003), NKp44 (Fregni et al. 2013),or increment of the $CD16^{-}$ population (Holtan et al. 2011; Bruno et al. 2013), have also been described as features associated with hyporesponsiveness. The downregulation of some of these receptors could be explained by continuous engagement and consequent internalization (Oppenheim et al. 2005; Sanchez-Correa et al. 2011), but modification of the mRNA levels of some of these receptors has also been described (Mamessier et al. 2011; Gillard-Bocquet et al. 2013) including decreased levels of mRNA for granzyme A and B (Al Omar et al. 2011) and perforin (Bajpai et al. 1991). Decreased levels of Eomes and T-bet transcription factors have been described for hyporesponsive NK cells, indeed forced overexpression

of Eomes was reported as inducing recovery of some NK cell functionality (Gill et al. 2012). However, other articles didn't find any difference in NK cell receptor expression (Verhoeven et al. 2008).

Other publications have described that sustained receptor engagement alters the formation of complexes between NK cell receptors and adaptor proteins, due to reductions in the expression of adaptor proteins such as CD3-ζ (Patankar et al. 2005; Ciszak et al. 2007), FcεRI-γ (Lai et al. 1996) or Lck (Lai et al. 1996). This phenomenon could be reversed after IL-2 treatment (Coudert et al. 2005).

Finally, NK cells isolated from tumour microenvironments have been proposed to secrete proangiogenic factors as VEGF, PIGF or IL-8 (Bruno et al. 2013), and the NK cell populations undergoing inactivation, have been reported to correspond to those cells able to form conjugates with target cells (Jewett and Bonavida 1995).

On the other hand, multiple articles studying NK cells from cancer patients have not observed any loss of NK cell function (Tursz et al. 1982; Caldera et al. 1992; Portner et al. 2012), or only lower cytotoxicity, but normal IFN-γ and TNF-α secretion (Carrega et al. 2008), despite in some cases the continuous expression of NK ligands (Buddingh et al. 2011). Indeed while in some cases the expression of, for example, NKG2D ligands, has been related with a poor prognosis in cancer (Li et al. 2009; McGilvray et al. 2010), in others expression of these molecules is associated with good prognosis (McGilvray et al. 2009; de Kruijf et al. 2012). The complexity associated with the diversity of individuals studied, and their tumours, the effects of heterogeneity in tumour microenvironments as well as the variety of ligands and NK cell receptors, generating different outcomes of NK cell mediated tumour immunosurveillance, cancer immunoediting and evasion, could explain why in some cases the outcome of immunosurevillance is tumour or virus elimination, while in others evasion or even immunosuppression takes place. This same range of factors may also explain the wide diversity of characteristics described as associated with NK cell unresponsiveness.

The thin line between self and non-self, the continuous tuning of fully pre-armed NK cells and the variegated phenotypes and characteristics described for hyporesponsive NK cells are probably all responsible for the fact that the question of which are the changes in the NK cell originated by target cells stimulation that modulates reactivity versus inactivity, still remains to be answered. A full understanding of the processes that contribute to the inability of NK cells to detect targets and cause this potentially dangerous "tolerization" is necessary in order to be able to prevent this phenomenon.

2.2 Unresponsive lymphocytes and calcium

Clemens von Pirquet in 1908 observed a transient loss of hypersensivity reaction to the tuberculin skin test associated with acute measles infection, and was the first to use the word anergy, to describe this situation where, in contrast to allergy, viruses induce a suppression of immunity (von Pirquet 1908). This word was used later by Nossal & Pike to name the functional inactivation of B cells triggered after the administration of high doses of antigen (Nossal and Pike 1980). Later the term was also used to describe a T cell hyporesponsive state after defective co-stimulation (Chai and Lechler 1997), and its usage has been extended to describe a variety of different phenomena of immune cell suppression affecting different types of cells including $CD4^+$ and CD8+ T cells, B cells or NKT cells (Schwartz 2003; Sullivan and Kronenberg 2005). With time, study of a range of different models of unresponsive states of lymphocytes has shown that many of them represent different biological situations of hyporesponsiveness with different characteristics (Schwartz 2003; Choi and Schwartz 2007). Some characteristics of these models are outlined below.

Anergy or **Clonal anergy** has been mainly studied for $CD4^+$ T cells and B cells. For T cells anergy has been described as a state induced in T cells following antigen encounter which renders the lymphocyte hyporesponsive, with no ability to produce IL-2 and other cytokines, and in growth arrest (Jenkins et al. 1990). In $CD4^+$ T cells it has been mainly studied after TCR strong stimulation in the absence of costimulation or after weak TCR stimulation, and can be reverted with IL-2 stimulation (Beverly et al. 1992). A predominant activation of the calcium/NFAT pathway and a blocking of the Ras-MAP kinase (Li et al. 1996) pathway have been identified as critical steps in anergy generation. In vitro, induction of anergy by calcium ionophores or mTOR inhibitors (Powell and Delgoffe 2010), and its blocking by cyclosporine A (Schwartz 2003), has been used to study the biochemical events related to this state.

Adaptive tolerance or *in vivo* anergy describes a process in which the T cell becomes desensitized when antigen stimulation persists (in contrast to clonal anergy where unresponsiveness is maintained even in the absence of antigen presentation). It is also characterized by a block in production of IL-2 and other cytokines by T cells accompanied by a block in proliferation. Moreover, it cannot be reversed with IL-2 stimulation (Rocha et al. 1993). These cells show a decreased ability to induce the Calcium-Calcineurin-NFAT pathway, but normal activation of Ras/MAPK signaling (Chiodetti et al. 2006) (which represents the opposite pattern to that described for anergic cells).

Activation-induced nonresponsiveness (AINR) or **exhaustion** is characterized by an inability to produce IL-2, but these cells can still be rescued by IL-2, and have been demonstrated to be able to survive in the absence of continuous antigen stimulation maintaining a stable phenotype (Wherry 2011). This process has mainly been studied in $CD8^+$T cells during chronic infections (Moskophidis et al. 1993) as well as in human cancer, although it has been described also in $CD4^+$T cells (Han et al. 2010). It appears at the peak of the $CD8^+$ T cell response after long exposure to antigen and has been suggested to act to control proliferation. These cells are described to have Ras/MAPK signaling defects (Tham and Mescher 2001;

Mescher et al. 2007). It is characterized by a progressive loss of effector functions, and of the proliferation of memory T cells, together with the upregulation of receptors such as PD-1, CTLA-4, Tim-3 or LAG-3, related to inhibitory pathways (Wherry 2011). These exhausted cells, could also exert cytotoxicity and proliferate in response to acute infections, and PD-1 blockade can rescue them (Utzschneider et al. 2013).

Activation-induced cell death (AICD) can be induced after repeated TCR stimulation, and it has been argued that this process acts to ensure tolerance and eliminate autoreactive T cells, or as a feedback mechanism to guarantee the termination of an ongoing immune response eliminating chronically activated T cells. It is mediated mainly by death receptors, and IL-2, IL-4 or IFN-γ play an important roles in AICD induction (Brenner et al. 2008). This process has also been described for NK cells, and CD16 mediated activation or IL-2 stimulation (Ortaldo et al. 1995) or even co-culture with target cells (Hasenkamp et al. 2010) can cause AICD on NK cells.

Suppression of immune cells can be also be mediated by regulatory T cells, the production of regulatory cytokines such as IL-10, IL-4 or TGF-ß, or nutrient and energy deprivation.

Alterations in calcium levels has been related to T cell anergy since 1987 (Jenkins et al. 1987), In those studies, Jenkins et al. demonstrated that increases of intracellular calcium levels triggered, in a dose dependent manner, by ionophores like ionomycin could establish an unresponsive state similar to anergy. From that moment ionomycin has been used as an anergy-inducing agent in $CD4^+$ T cells by several groups (Macian et al. 2002). Ionomycin is a calcium ionophore which causes an influx of external calcium and the leak of intracellular Ca^{2+} stores, which will activate the Calcium-Calcineurin-NFAT cellular pathway. However, ionomycin generated anergic $CD4^+$ T cells gradually regain (after 4 days) their proliferative and IL-2 production abilities, and the ionomycin treated cells not only lose their ability to produce IL-2, but also other cytokines, so the characteristics of this phenomenon are somewhat different from other models of anergy (Schwartz 2003). Ionomycin has been used to study anergy in other cell types such as NKT cells (Kojo et al. 2009). Increased intracellular calcium basal levels (Cooke et al. 1994) and deregulated use of NFAT transcription factors is also observed in tolerant B cells generated by chronic self-antigen stimulation (Healy et al. 1997).

As discussed previously, Natural killer cell activation is regulated by a balance of signals from different activating and inhibitory receptors. The activating NK receptors, with the exception of CD16, act as co-receptors so that NK cells require the cooperation of different signals to initiate an effector response and a potent Ca^{2+} flux. Different signaling pathways have been described to be preferentially activated via different families of receptors due to the use of different adaptor molecules. For example, NCRs which signal through ZAP-70 and Syk, trigger LAT phosphorylation, activating MAPK, JNK and Calcium-NFAT signals, while the 2B4 receptor also activates LAT via SAP and Fyn signaling. In contrast, the NKG2D receptor via DAP10 directly activates PI3K-JNK and MAPK pathways (Lanier 2008).

The necessity for synergy between different families of receptors could indicate that receptors need to cooperate to achieve a sufficient quantity of positive signals, or that they cooperate to have access to the activation of different signaling pathways engaged by each receptor. It is important to note that different combinations of receptors will cause synergistic activations, but not all of them: two NCRs would not synergize, implying that they are able to achieve some level of activation but never as much as the synergy between NCR or 2B4 with NKG2D receptor. In any case, contrary to what happens in T cells where free Ca^{2+} concentrations increase in response to TCR engagement, single stimulation through NK cell receptors, except CD16, induce no or weak intracellular Ca^{2+} mobilization, whereas those combinations able to synergize induce a strong enhancement in free Ca^{2+} inside the cell, that correlates with cytotoxicity and cytokine secretion (Bryceson et al. 2006). Thus calcium only stimulation of NK cells mediated by ionomycin could then mimic a chronic stimulation mediated by combinations of different activating receptors or imbalanced stimulation.

The characteristics of hyporesponsive NK cells are very diverse as described in the previous section, sharing some of their properties with other unresponsive lymphocytes.

	NK cell poresponsiveness	Clonal anergy	Adaptive anergy. Tolerance	AINR. Exhaustion	AICD
Caused by suboptimal activation	? Suboptimal inhibition? (licensing)	Yes	No	No	No
Caused by chronic optimal activation	Yes	No	Yes	Yes	Yes
Continous stimulation required	No	No (Quill and Schwartz 1987)	Yes (Rocha et al. 1993)	No (Tham and Mescher 2001)	-
IL-2 driven recovery	Yes/No	Yes (Beverly et al. 1992)	No (Rocha et al. 1993)	Yes (Tham and Mescher 2001)	-

In order to gain insight into the phenomenon of hyporesponsiveness in NK cells, we wanted to establish a simple, reproducible model which could help us to understand better the processes that lead to induced NK cell hyporesponsiveness. We decided to use overnight stimulation with the calcium ionophore ionomycin to simulate a continuous NK cell stimulation, as this strategy has previously been productively used to study $CD4^+$ T cell anergy (Macian et al. 2002).

Objectives

Different characteristics have been described for unresponsive NK cells. However, there is no hint about the mechanisms that allows the generation and maintenance of this state. The induction of a calcium flux and its maintenance is one of the first steps during NK cell activation. We propose the use of a sustained calcium flux as a hyporesponsiveness inducing method for NK cells, as an approach to mimic chronic activation by stimuli.

To study the influence of maintained calcium activation on NK cells we proposed the following objectives:

1. Study the impact of ionomycin treatment on activated and freshly isolated NK cells.
    1.1 Analyze if ionomycin treatment is able to induce an unresponsive state on NK cells at cytotoxic and cytokine production levels.
    1.2 Optimize ionomycin treatment protocol for NK cell unresponsiveness induction.
    1.3 Evaluate the reversion of the hyporesponsive state by cytokine stimulation.
    1.4 Characterize differential receptor expression on unresponsive NK cells.

2. Examine at mRNA levels, the consequences of ionomycin treatment on NK cells, and compare these differences with unresponsive NK cells or other unresponsive lymphocytes.

Materials and methods

1. NK cell isolation

NK cells were isolated from buffy coats, donated by healthy volunteers, obtained from the "Centro de Transfusiones de la Comunidad de Madrid". Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque (GE Healthcare) gradient centrifugation. Briefly, buffy coats were diluted with PBS 1:3, layered on top of Ficoll in a 2:1 proportion and centrifuged for 30min at 400 g. PBMCs, found at the interface between the plasma and the Ficoll, were harvested and washed. NK cells were further isolated and purified from PBMCs using a magnetic bead isolation kit by negative selection (Human NK isolation kit, Miltenyi Biotec) according to the manufacturer instructions.

After isolation, NK cells were cultured in the presence of irradiated (40 Gray) Daudi and RPMI-8866 as feeder cells, and irradiated autologous PBMCs in Roswell Park Memorial Institute medium (RPMI 1640, Lonza) supplemented with 10% FBS, 10% Human Serum (Sigma Aldrich), 50 U/mL rhIL-2 (Peprotech) the first week and later with 5% FBS, 5% Human Serum, 50 U/mL rhIL-2. This media was supplemented with 4mM L-glutamine, 0.1mM non essential amino-acids, 10mM HEPES, 1mM sodium pyruvate, 100 U/mL penicillin, 100 U/mL streptomycin and 50µM ß-mercaptoethanol. The NK cells were restimulated and diluted weekly during one month, and only if the culture was >85% pure NK cells.

Resting NK cells (rNK) were isolated by negative selection from leukapheresis from healthy donors provided by the NIH department of transfusion medicine.

2. Cell lines

All cell lines were maintained at 37°C and 5% CO_2 in a humidified incubator and split as necessary. The cell lines RMPI 8866, Daudi, K562, 721.221, P815 and Jurkat were maintained in RPMI medium and the 293T cells were cultivated in Dulbecco's Modified Eagle medium (DMEM). All media were supplemented with 10% heat-inactivated FBS, 2mM L-Glutamine, 0.1mM non essential amino-acids, 10mM HEPES, 1mM sodium pyruvate, 100 U/mL penicillin, 100 U/mL streptomycin and 50µM ß-mercaptoethanol.

3. Ionomycin treatment of NK cells

NK cells were washed and resuspended at $1x10^6$ cells/mL in RPMI 10% FBS (without human serum or IL-2). Cells were then treated with either Ionomycin 1 μM (Sigma) or DMSO (Sigma), as vehicle control, and cultured for 16 hours. Control and ionomycin-treated cells were then washed and allowed to rest for 24 hours at $2x10^6$ cells/mL in RPMI 10% FBS. Where indicated, during this rest day, cell cultures were supplemented with 50U/ml IL-2 (Peprotech), IL-12 10ng/mL (Peprotech), IL-18 10ng/ml (MBL) or 100/1000 U/mL IFN-α (Peprotech).

4. Flow cytometry analysis

Live NK cells were stained with fluorescently labeled or unlabelled antibodies followed by PE or FITC fluorophor labeled F(ab')2 fragments of goat anti-mouse immunoglobulin (Dako). For staining, cells were washed and incubated in a PBS / 0.5% (w/v) Bovine serum albumin / 1% (v/v) Fetal bovine serum / 0.1% Sodium azide buffer (PBA buffer) with the antibodies specified in each particular experiment using the amounts recommended by the manufacturer. When the staining was finished, cells were washed twice with PBA buffer. All the staining was performed on ice, and the labelled cells were maintained on ice until analysis.

For intracellular staining, cells were first stained with antibodies on ice, washed with PBA and then fixed using 2% para-formaldehyde (PFA) fixation buffer for 10 minutes at room temperature in the dark. After fixation, cells were washed twice with PBA, and resuspended in PBA containing 0.5% Saponin (permeabilization buffer) and incubated with the desired antibodies at room temperature and in the dark. When the staining was finished, cells were washed twice with permeabilization buffer and once with PBA.

For staining cytotoxic granules, the cells were washed twice with PBS and fixed with 70% EtOH, adding the EtOH dropwise while vortexing. Samples were kept overnight at -20°C. For staining, cells were washed twice in abundant PBS at 1,700 rpm. Cells were then stained as before with primary and secondary antibodies in PBA buffer.

Cells were analyzed using a FACScalibur Becton Dickinson cytometer (BD Biosciences), Cytomics FC500 (Beckman Coulter) or Gallios (Beckman Coulter) depending on the experiment. Data were analyzed with either Kaluza Flow Cytometry Analysis v1.2, Summit V4.3.02 or FlowJo V9.6.2 software.

The antibodies used are detailed below:

Molecule	Clone	Supplier
CD2-APC	LT2	Immunotools
CD9	MEM-192	V. Horejsi (Institute Molecular Genetics AS AR)
CD11a	HI111	BioLegend
CD11a open	NK1-L16	C.G.Figdor (Nijmegen Centre for Molecular Life Sciences)
CD11-CD18	Ab24	Abcam
CD11b	ICRF44	BioLegend
CD11b activated	CBRM1/5	Biolegend
CD11c-APC	BU15	Immunotools
CD16	B73.1	J.L. Strominger (Harvard University)
CD18 ascites	LIA3/2	F.Sánchez-Madrid (H. La Princesa)
CD25-FITC	HI25a	Immunotools
CD27-PE	LT27	Immunotools
CD28	CD28.2	BD Pharmingen
CD29-FITC	MEM-101A	Immunotools
CD44-APC	MEM-85	Immunotools
CD45-FITC	MEM-28	Immunotools
CD49d-FITC	BU49	Immunotools
CD53-FITC	HI29	Immunotools
CD54(ICAM1)-FITC	1H4	Immunotools
CD56 unlab/-PE	MEM-188	BioLegend
CD58-PE	MEM-63	Immunotools
CD59-APC	MEM-43	Immunotools
CD61-FITC	C17	Immunotools
CD62L-APC	LT-TD180	Immunotools
CD69	FN50	BD Pharmingen
CD85j (LILRB1)	GHI/75	BD Pharmingen
CD94-PE	HP-3D9	BD Pharminegn
CD95	IM2446	Immunotech
CD107a-APC	H4A3	BioLegend
CD152-PE (CTLA4)	BN13	Immunotech
CD158a (KIR2DL1)	EB6	Immunotech
CD158b (KIR2DL2/DL3)	GL183	Serotec
CD158e1 (KIR3DL1)-PE	DX9	B&D Pharmingen
CD161 (KLRB1)	HP-3G10	M. López-Botet (Universidad Pompeu Fabra)
CD244 (2B4)	2-69	B&D Pharmingen
DNAM1	MAB666	R&D
HLA-ABC-PE	W6/32	Immunotools
HLA-DR-PE	LT-DR	Immunotools
IFN-γ - PE	4S.B3	Biolegend
Mouse IgG1	MOPC-21	Sigma

NKG2D	MAB139	R&D
NKp30	210845	R&D
NKp46	195314	R&D
Perforin	dG9	Biolegend
PD1	EH12.2H7	BioLegend
ß-actin	AC-15	Sigma
Annexin-V-FITC	731725	Beckman
7-AAD		
Propidium iodide	660755	Beckman

5. Degranulation assay

NK cell degranulation was analyzed following the 2 hour protocol described in (Bryceson et al. 2010). This assay quantifies secretory lysosome release by measuring changes in the cell surface expression of the transmembrane protein Lamp1 (CD107a) which resides in secretory lysosomes, but upon activation becomes incorporated into the NK cell surface as lytic granules fuse with the NK cell membrane. NK cells were coincubated with K562 target cells (or other target cell lines when indicated) for 2 hours in round bottom 96 well plates, in an effector to target cell (E:T) ratio of 1:2. (generally 100,000 NK cells and 200,000 targets were used). The co-culture was harvested and stained with anti-Lamp1*APC and anti-CD56*PE antibodies for 30 minutes and the percentage of CD56 positive - Lamp1 positive cells quantified by flow cytometry. Where indicated the cells were stimulated with PMA 100ng/ml (Calbiochem) / Ionomycin 0.5 μM (Sigma) as a positive control.

6. Cytotoxicity assay

Cytotoxicity was assessed by the analysis of Propidium Iodide (PI) (Beckman) positive K562 or Jurkat cells, after coculture with NK cells for different time points, following the protocol described in (Slezak and Horan 1989) and based on the observation that only dead cells stain with PI as this compound is membrane impermeant. Briefly, target K562 or Jurkat cells were stained with CFSE (Invitrogen) membrane dye following the recommendations of the manufacturer. Target cells were then cocultured with NK cells at a 3:1 E:T ratio for 1h, 3h or 5 hours in 96 well round bottom plates (generally 250,000 NK : 75,000 Targets were used). Target cells and effector cells cultured alone were used to measure spontaneous cell death. The cocultures were collected and stained with 100μg/mL PI during 10 minutes. Samples were analyzed by flow cytometry. CFSE only positive cells represent live target cells, while CFSE & PI double positive cells were scored as dead target cells.

For quantifying target cell death the following formula was used:

$$\% \text{ target cell death: } \frac{\% \text{ CFSE positive \& PI positive}}{\text{CFSE positive cells}} \times 100$$

7. Redirected antibody dependent cytotoxicity

FcγR-positive P815 cells are not normally susceptible to NK cell lysis, but when incubated with antibodies specific for NK cell activating receptors, these antibodies bind to the P815 cells via Fc/FcγR interactions and to the NK cell via the antibody variable regions to act as a bridge that triggers NK cell cytotoxicity via specific receptors. They were cocultured with NK cells in the presence of different combinations of antibodies at 5μg/mL for 2 hours, following the same "Degranulation assay" described previously, or 1, 3 and 5 hours, following the "Cytotoxicity assay" described earlier. As controls, P815 cells loaded with MOPC-21 (Mouse IgG1 monoclonal isotype control antibody) and P815 cells that had not been incubated with antibody were used. Before staining with anti-CD56*PE and anti-Lamp1*APC antibodies, unoccupied P815 Fc-receptors were blocked with 10% mouse serum. Samples were analyzed by flow cytometry.

8. Measurement of the IFN-γ production

IFN-γ production was analyzed by flow cytometry following the 6 hour protocol described in (Bryceson et al. 2010). NK cells were coincubated with target cells for 6 hours in a 96 well round bottom plate, in a E:T ratio of 1:2 (generally 150,000 NK : 300,000 Targets were used), in the presence of 2,5 μM Monensin (Sigma) which blocks exocytosis and so leads to the intracellular retention and accumulation of IFN-γ for subsequent detection of producer cells by intracellular flow cytometry. Briefly, the cells were fixed with ice cold 2% para-formaldehyde (PFA) fixation buffer for 10 minutes and after washing, cells were permeabilized with 0.5% Saponin (permeabilization buffer) and and stained with anti-IFNγ*PE antibodies for 30 minutes and quantified by flow cytometry the percentage of IFN-γ positive cells.

9. Conjugate formation assay

The formation of stable conjugates between K562 target cells and NK cells was analyzed using the two color flow cytometry method described in (Burshtyn and Davidson 2009). Briefly K562 target cells were stained with the green fluorescent dye PKH2 (Sigma) while NK cells were labeled with the red dye PKH26 (Sigma), following the recommendations of the manufacturer. NK cells were dispensed in different FACs tubes depending on the number of time-points to analyze, and target cells were added

in an E:T ratio of 1:2 (generally 100,000 NK : 200,000 Targets were used). Cells were centrifuged at 4°C and 100g for 1 minute to initiate the cell:cell interactions and the tubes were incubated for the desired time points at 37°C to allow the formation of conjugates, tubes were then vortexed for 3 seconds to separate weakly attached cells and immediately fixed with 4% ice cold PFA (final concentration 1%). Control samples containing NK and target cells alone were also fixed. One NK-Target cell coculture control tube was kept at 4°C during the length of the experiment and fixed when the experiment was finished. Finally, the PKH26 and PKH2 double positive events were quantified as conjugates, and the percentage of NK cells forming conjugates calculated as follows:

$$\% \text{ conjugates: } \frac{\% \text{ PKH2-PKH26 double positive events}}{\% \text{ PKH26 positive cells}} \text{ X } 100$$

10. Analysis of cell viability

NK cells were stained with CD56*PE and Lamp1*APC antibodies. After staining, cells were washed first with PBA and then with cold PBS, before being stained with Annexin-V FITC (Beckman) and 7-AAD (7-Aminoactinomycin D) in Annexin Binding buffer (Invitrogen) following the manufacturer instructions. Cells were incubated 15 minutes on ice and analyzed by flow cytometry. Annexin-V stains phosphatidylserine (PS), which appears on the cell surface in non-permeabilized cells in early apoptotic states when cells have lost their PS asymmetric distribution. 7-AAD has high affinity for DNA and intercalates with it, and as it is membrane impermeable, only stains cells with disrupted membranes, such as cells from late apoptotic states.

11. Calcium flux measurement

NK cells were stained with 4μg/mL Fluo-4 AM (Life technologies F-14201) and 10 μg/mL FuraRed AM (Life Technologies F-3021) dyes in HBSS with calcium and magnesium, supplemented with 1% FBS and 4mM Probenecid during 30 minutes at 37°C. Cells were washed and kept on ice until experiment. For antibody stimulation, after dye loading, NK cells were stained with primary antibodies on ice during 30 minutes. Excess antibody was washed away and cells were resuspended in HBSS with calcium and magnesium plus 1% FBS. Cells were incubated at 37°C during 5 minutes prior to acquisition of the data in the flow cytometer. For stimulation, after 30 seconds of acquisition to establish a baseline, either ionomycin, to a final concentration of 0.5 μM or Goat anti-mouse F(ab')2 (added to a final concentration of 10 μg/mL to cross-link the primary mAb) were added. Events were acquired, using a FACScalibur Becton Dickinson cytometer, for 5 minutes, and then analyzed with FlowJo software (Treestar, Ashland,

OR, USA. Version 9.6.2). Monoclonal antibodies used to stimulate the different NK cell receptors, either individually or in combination, were NKG2D (clone 149810 R&D), NKp46 (clone BD 9E2), 2B4 (clone C1.7) or CD56 (clone BD B159) as a negative control. Where indicated 1mM EGTA was added 5 minutes 37°C prior to data acquisition to chelate calcium from the media and so be able to follow the release of Ca^{2+} from intracellular stores only. To study the influence of the actin cytoskeleton on NK cell responsiveness to receptor crosslinking by mAbs, Latrunculin was added, to a final concentration of 1μM, during the 5 minutes at 37°C prior to data adquisition.

12. Gene expression analysis by microarray

Gene expression experiments were done in collaboration with the Genomics Unit of the CNB. After the ionomycin treatment protocol, total RNA was isolated by TRIZOL reagent (Invitrogen) according to the manufacturer's instructions, and subsequent purification by RNA column cleanup (Qiagen RNeasy). A minimum of 4μg of RNA was used for the gene expression analysis by microarrays. The following steps were performed by the CNB-Genomic Unit. Once RNA quality was confirmed by Bioanalyzer2100, cDNA was synthesized and labeled with Cy3 or with Hyper5 Mono NHS ester (DMSO treated cells vs Ionomycin treated cells). The whole genome transcriptional profile was determined by hybridization with the two color AGILENT human gene expression 4x44K v2 microarray kit (Agilent G4845A) which analyzes the expression of 27,958 genes. Data were collected, and preprocessed for further analysis. Finally data were visualized using the FIESTAviewer v.1.0 software (http://bioinfogp.cnb.csic.es/tools/FIESTA/index.php). Differentially expressed genes were selected from the FIESTAviewer when they show a False Discovery Rate (FDR) < 0.05 by Rank Products analysis, and a Fold Change <= -1.6 (repressed genes) or >= +1.6 (induced genes).

Differentially expressed genes were subjected to deeper analysis using different online tools (*e.g.* Babelomics-Genecodis, Panther, g:Profiler), and compared with published data sets of differentially expressed genes under different treatments by Venn diagrams, as indicated. Quantative RT-PCR was used to confirm the differential expression of genes of interest identified in the cDNA microarray experiments.

13. Quantitative RT–PCR

Total RNA was extracted with the TRIZOL reagent (Invitrogen). cDNA was prepared using 1μg of this RNA by retrotranscription with Random Hexamers (Roche) and SuperScript II Reverse Transcriptase (Invitrogen) in the presence of RNAse inhibitors (Promega), in a final volume of 20 μL, according to the manufacturer's instructions.

qPCR reactions were carried using Fast Plus EvaGreen® qPCR Master Mix (Biotium) in a final volume

of 8 µL. 18S levels were used as controls.

The primers used are detailed below:

GENE	Forward primer	Reverse primer
dap10	5′- GCACTTCAGGCTCTTGTTC	5′- GCCTGGCATGTTGATGTAGA
itgb2	5′- CAAGCTGGCTGAAAACAACA	5′- ATTGCTGCAGAAGGAGTCGT
itgam	5′- GCCGGTGAAATATGCTGTCT	5′- GCGGTCCCATATGACAGTCT
myom2	5′- GAACCCCACAATTCCTCTGA	5′- TCGATCCATCACCATCTCAA
gzmh	5′- CAGAAGGACTGCCAGTGTGA	5′- ACTCCTGGAGGTGTCCCTTT
cd300a	5′- GTCCTGCAAACCTCAGCTTC	5′- CCCACTGCAAACAGGGTAGT
il18r1	5′- TAGTGCCTGGAGGAGCTGTT	5′- ATTGGGGCAAGAATGTGAAG
egr1	5′- TGACCGCAGAGTCTTTTCCT	5′- TGGGTTGGTCATGCTCACTA
ncr3	5′- CCTGAGATTCGTACCCTGGA	5′- GAAACGGGAAGAAGCAAGTG
ctsw	5′- CCACCCCAAGAAGTACCAGA	5′- GTGGCCTTGATCACACCTTT
sell	5′- AAACCCATGAACTGGCAAAG	5′- CGCAGTCCTCCTTGTTCTTC
matk	5′- TACCGCGTCAAGCACCACACCAG	5′- CACTCTCCACTCTCTCGGTCCTCTG
cd56	5′- TATTTGCCTATCCCAGTGCC	5′- CATACTTCTTCACCAACTGCTC
gnly	5′- TCTCTCGTCTGAGCCC	5′- GCAGCATTGGAAACACT
id2	5′- CGGATATCAGCATCCTGTCC	5′- TCATGAACACCGCTTATTCAG
18s	5′- GGGACTTAATCAACGCAAGC	5′- GCAATTCCCCATGAACG
ß-*actin*	5′- CCCAGCACAATGAAGATCAA	5′- CGATCCACACGGAGTACTTG

PCR conditions were as follows: 50°C for 10′, 95°C for 2′, 40 cycles at 95°C 15′′ and 60°C 1′. Dissociation analysis was performed at the end to confirm the specificity of the reaction This program consisted of 15′′ at 95°C, 15′′ at 60°C and a gradual increase to 90°C for 20′, during which time fluorescence was measured. Reactions were run with the Applied Biosystems 7900HT system.

Samples for each experimental condition were analysed in triplicates. Purified water was included as a non-template control. The relative expression of each gene was calculated from the fluorescence increase data *vs.* cycle number, from which the Ct value is obtained; after correction for 18S control amplification, the ΔCt is obtained. Finally, the sample is compared with a reference sample, and the relative expression of the gene is calculated by applying the equation $2^{-(\Delta\Delta Ct)}$ to obtain the relative quantification as relative quantity (RQ).

14. Western blotting

Cells were lysed in ice-cold lysis buffer (1% Igepal CA-630, 50mM Tris pH 7.5, 150mM NaCl, 5mM EDTA) with protease inhibitors (1µM Pepstatin A, 1µM Leupeptin and 0.5mM Iodoacetamide) for 30min on ice. Lysates were then centrifuged (13,000 rpm for 5min) to pellet nuclei and the protein

concentration of the supernatant quantified using the Coomassie protein assay reagent (Thermo Scientific) before analysis by SDS-PAGE and Western Blot. 2xloading buffer (125mM Tris pH6.8, 4% SDS, 20% glycerol and bromophenol blue) with or without 100mM DTT (reducing / non reducing conditions) was added to lysates.

Proteins were separated in acrylamide gels (Electrophoresis running buffer: 195mM Glycine, 24.8mM Tris base, 0.1% SDS) and then transferred onto activated polyvinylidene fluoride (PVDF) transfer membranes (Immobilon-P. Millipore) using a semi-dry electroblotting system (BioRad) in transfer buffer (25mM Tris base, 192mM Glycine, 20% methanol), and blocked with a solution containing TBS (100mM NaCl, 10mM Tris Base) and 0,05% Tween20 (TBS-T) with 5% non-fat milk for 45 minutes. After washing, the membrane was incubated with appropriate primary antibody diluted in TBS-T overnight at 4°C. The following day, membranes were washed and incubated with secondary antibodies labelled with Horseradish Peroxidase (HRP) for 60 min. The membranes were then washed and bound antibody visualized by reaction with Amersham ECL western blotting detection reagents (GE Healthcare) and X-ray films (Konica Minolta).

The primary antibodies used are detailed below:

Molecule	Clone	Supplier	Dilution
CD3 ζ	488	Dr. Balbino Alarcón (Risueno et al. 2008)	1:5,000
ß-actin	AC-15	Sigma	1:15,000

15. Confocal microscopy

Borosilicate microscopy cover glasses were coated with 25µg/mL Poly-L-lysine (Sigma) for 30 minutes at room temperature (RT). After washing with PBS, NK cells alone or NK cell/K562 cocultures (1:1 ratio) were applied. When K562 were used, they were stained previously with CMAC (Molecular Probes) to distinguish between NK cells and K562. After 1 hour to allow cell attachment to the Poly-L-Lysine coated slides, each coverslip was washed and fixed with 4% PFA during 15 minutes. For intracellular staining cells were permeabilized with PBS containing 0.3% Triton X-100. Samples were blocked with 10% Human Serum (HS). Antibodies were diluted in 1% HS-PBS for extracellular staining or 1%HS-PBS-0.3%Triton if intracellular staining was necessary. Cells were stained with primary antibody for 45 minutes at RT, washed and stained with secondary antibody for another 45 minutes at RT. For F-actin staining, Phalloidin-Alexa 488 was used and incubated together with the secondary antibodies.

The secondary antibodies used were GAM-Alexa 568, GAM-Alexa 488, GAM-Alexa 648, GAR-Alexa 546 all from (Invitrogen). Coverslips were carefully mounted using 15µL Prolong-Gold Antifade Reagent (Invitrogen) and the sample was allowed to cure in the dark at room temperature for 24 hours. Finally, each coverslip was sealed at the corners with nail polish to prevent the coverslip from moving. Before inspection, the samples were stored in dark at 4°C. Coverslips were visualized using the Olympus Fluoview 1000 or the Leica TCS SP5 confocal microscope. Images were analyzed using ImageJ software and its Fiji image processing package (Schindelin et al. 2012).

Reagents used are detailed below:

Primary antibodies	CD11a (mouse)	HI111	Biolegend
	Perforin (mouse)	dG9	Biolegend
	Pericentrin (rabbit)	4488	Abcam
Secondary antibodies	Goat anti mouse Alexa Fluor 568		Invitrogen
	Goat anti mouse Alexa Fluor 648		Invitrogen
	Goat anti rabbit Alexa Fluor 546		Invitrogen
Phalloidin-Alexa Fluor 488	Invitrogen		

16. Statistical analysis

Data are represented as the mean values, and error bars show Standard deviations. Statistical analysis was done with the GraphPad software (Prism). Student's *t*-test or Mann-Whitney test was performed to compare pairs of data. Multiple data were compared with one-way analysis of variance (ANOVA) and Tukey-Karmer post-test. When two variables were analyzed, two-way ANOVA and Bonferroni post-test were used. When the p-value < 0.05, differences were considered statistically significant. p-values are indicated by asterisks as follows:* $p<0.05$; ** $p<0.01$; *** $p<0.001$.

List of reagents

Reactive	Supplier	Reference
30% Acrylamide/Bis solution	Biorad	161-0156
Amersham ECL	GE Healthcare	RPN1231
BX795	Invivo Gen	tlrl-bx7
CFSE	Invitrogen	C34554
CMAC	Molecular probes	C2110
Coomasie protein assay reagent	Thermo Scientific	23200
DMSO	Sigma	D2650
DNPs	Roche	11969064001
Ficoll-Paque	GE Healthcare	17-1440-02
Human NK cell isolation kit	Miltenyi Biotech	130-092-657
Human serum	Sigma	H4522
IFN-α	Peprotech	300-02A
Igepal-CA630	Sigma	I7771
Iodoactamide	Sigma	I612
Ionomycin	Sigma	I9657
Leupeptin	Calbiochem	108975
Monensin	Sigma	M5273
Paraformaldehyde	Sigma	158127
Pepstatin A	Calbiochem	516481
PKH2	Sigma	PKH2GL
PKH26	Sigma	PKH26-GL
PMA	Calbiochem	524400
Poly-L-Lysine	Sigma	P4832
Fast Plus EvaGreen® qPCR Master Mix	Biotium	31020
Prolong Gold	Invitrogen	P36930
Propidium iodide	Beckman	660755
Protamine sulfate	Sigma	P4020
Puromycin	Calbiochem	540411
PVDF transfer membranes	Millipore	IPVH00010
Random Hexamers	Roche	58002113-01
rh IL-12 p70	Peprotech	200-12
rh IL-18	MBL	B001-5

rh IL-2	Peprotech	200-02
RNAse inhibitors	Promega	N251A
Saponin	Sigma	S7900
SuperScript II RT	Invitrogen	18064-022
T4 DNA ligase	New England Biolabs	M0202S
Triton X-100	Sigma	T9284
Trizol Reagent	Invitrogen	15596-026
Tween-20	Sigma	P5927
X-ray films	Konica Minolta	

Results

1. Establishment of an in vitro system to study NK cell hyporesponsiveness

As described in the introduction, previous studies analyzing NK cell induced hyporesponsiveness have been performed using transgenic mice, NK cells isolated from patients with different types of cancer or suffering virus infections, or cocultures of NK cells with target cells during prolonged times. The main problem of these approaches is the use of a non-human mammal system in the case of transgenic mice, or the low numbers of available cells when working with patient-derived samples. The coculture strategy allows the generation of large numbers of induced-hyporesponsive NK cells but different receptor-ligand interactions between NK cells and different target cells may induce different effects.

In view of the extensive heterogeneity in receptor-ligand interactions as well as the variability between donors, and with the hypothesis that different strategies used by target cells to induce NK hyporesponsiveness, will probably at the end affect the same processes or produce similar changes in NK cells, we decided to establish a model based on pharmacological induction of NK cell hyporesponsiveness.

Different chemicals have previously shown to affect NK cell functions *e.g.* histone deacetylase inhibitors (Ogbomo et al. 2007), demethylating agents (Gao et al. 2009), glucocorticoids (Krukowski et al. 2011), statins (Raemer et al. 2009) or haemaglutinin (Mao et al. 2010). We decided to study the impact of ionomycin on NK cells as a way to mimic chronic exposure to stimuli, as it has been extensively used for study of $CD4^+$ T cell anergy (Macian et al. 2002), with the aim to characterize some of the processes underlying hyporesponsiveness in NK cells.

1.1 Optimization of the Ionomycin treatment protocol

Ionomycin has been used as an anergy inducing agent in $CD4^+$ T cells from 1987 (Jenkins et al. 1987) as it stimulates the Ca^{2+}-Calcineurin-NFAT pathway mimicking the T-cell receptor signaling (Signal 1) in the absence of costimulation (Signal 2) (Lafferty and Cunningham 1975), a combination which results in the induction of hyporesponsiveness (Schwartz 2003). Using this *in vitro* model Anjana Rao's lab and others described the genetic program, and biochemical changes that lead to $CD4^+$ T cell anergy (Macian et al. 2002; Heissmeyer et al. 2004). In those experiments $CD4^+$ T cells were resuspended at 1million cells/mL and treated with 0.5 or 1µM ionomycin, in an overnight culture (16 hours). To study the effect of ionomycin on NK cell function, NK cells maintained *in vitro* with feeder cells and IL-2, were washed and resuspended at a concentration of 1 million cells/ml in RPMI 10% FBS in the absence of IL-2, and treated with ionomycin 1µM (or DMSO, vehicle control) for 16 hours as previously used with $CD4^+$ T cells. After this treatment, cells were washed and rested for 24 hours at 2 million cells/mL in RPMI 10% FBS before coculture

with K562 target cells for 2 hours at an Effector:Target (E:T) ratio of 1:2. K562 is a classical NK cell target cell line that does not express MHC class I molecules, but does express activating ligands such as ULBP1 and ULBP2 (Fernandez-Messina et al. 2011). Immune recognition by NK cells was analyzed by assessing the ability of these cells to degranulate on contact with the K562 target cells by quantifying secretory lysosome release by the increased cell surface expression of the transmembrane protein Lamp1 (CD107a) which, as lytic granules are released, becomes incorporated into the NK cell surface **(**Fig. 1-A**)**. An approximately 40% (-39.85 +/- 13.93) decrease in the degranulation of the NK cells treated with ionomycin was observed, confirming that ionomycin treatment had a negative effect on NK cell function.

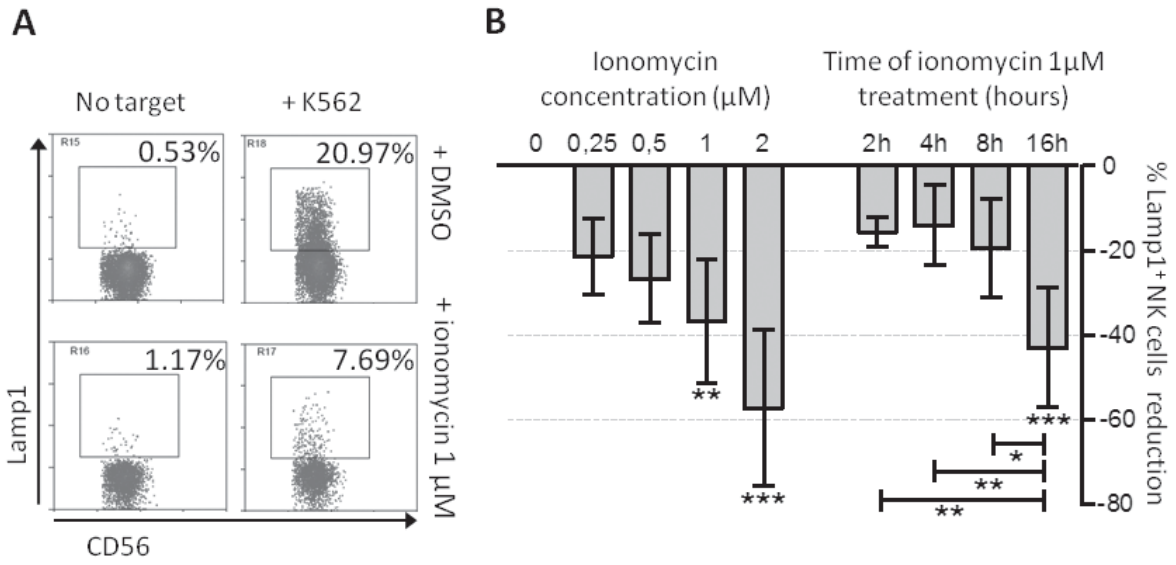
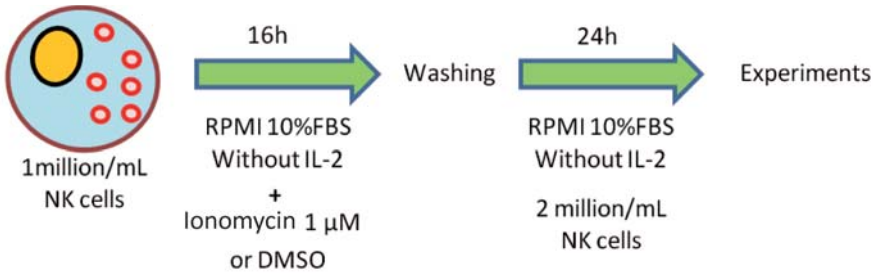


Fig. 1: Ionomycin treatment protocol optimization. (A) Example and scheme of the flow cytometry analysis of Lamp1 expression on NK cells after coculture with K562. **(B)** NK cells were treated with increasing amounts of ionomycin (from 0.25µM to 2µM). Treatment of NK cells with 1µM ionomycin was also titrated along time (from 2 hours to 16 hours). Cells were washed and rested for 24 hours. To evaluate the influence of ionomycin treatment, cells were cocultured with K562 target cells during 2hours, and the percentage of $Lamp1^+$ NK cells analyzed by flow cytometry.(n=5). The data show mean +/- SD. One way ANOVA and Bonferroni post-test analysis was used to calculate statistical significance using the logarithms of crude data. *p<0.05, **p<0.01, ***p<0.001.

After establishing that exposure to ionomycin had a negative impact on NK cell degranulation ability, the concentrations needed and the kinetics of this effect were defined to optimize the *ionomycin induced NK cell loss of function* protocol. To assess the minimum amount needed, ionomycin was titrated over a range of concentrations from 0.25µM to 2µM. The maximum effect was achieved after 2µM treatment but this was accompanied by a decrease in NK cell viability. Thus, furhter experiments were carried out using a concentration of 1µM. The necessity for an overnight culture (16 hours), was also studied by comparing the effects of 2, 4, 8, 16 hours of 1µM ionomycin treatment on NK cell function. The highest reduction in the NK cell degranulation capacity was observed after 16 hours of treatment **(**Fig. 1-B**).** The difference in response between 8h (-19.48% +/- 11.48 lower response

compared with control) and 16 hours (-42.95% +/- 14.09%) of ionomycin treatment was surprising. This necessity for a prolonged treatment suggests that novel protein synthesis processes are involved in the ionomycin induced NK cells loss of response. In summary, the induction of NK cell hyporesponsiveness after ionomycin treatment was dose and time-dependent.

The final protocol used for studying the effect of ionomycin on NK cells was as follows:



Scheme 2: Diagram of the protocol used ito study the *ionomycin induced NK cell loss of function*

During the course of the experiments outlined in this thesis, this degranulation experiment was performed prior to further experiments, to confirm that the ionomycin treated cells to be used were in a hyporesponsive state. A few cell lines almost disappeared by cell death after treatment with ionomycin 1µM, whereas the responsiveness of a few other cell lines was not affected by ionomycin 1µM, and higher amounts of the calcium ionophore had to be used for them to become hyporesponsive. All data shown were obtained using NK cells treated with ionomycin 1µM, and using only those cells that showed a reduced degranulation response after this treatment.

1.2 The cytotoxic function of Ionomycin treated NK cells

To cause apoptosis on target cells, NK cells secrete preformed cytotoxic granules which contain pore forming proteins and several proteases. NK cells can also release members of the TNF-α family including Fas-Ligand, TNF-α or TRAIL which activate the death receptor pathway by binding to the target cell. To study the ability of NK cells to kill target cells by these mechanisms, cytotoxic granule release was analyzed by assessing the induction of surface Lamp1 on NK cells, while the induction of target cell death (caused by both mechanisms) was followed by flow cytometry (propidium iodide staining).

1.2.1 The effect of ionomycin treatment on NK cell degranulation

NK cell lytic granules, upon target cell recognition, are polarized and secreted to the immune synapse formed between the NK cell and the target cell. To study cytotoxic granule release ionomycin treated NK cells and control NK cells were cocultured with K562 target cells in a E:T ratio of 1:2 during 2 hours and then, the presence of Lamp1 on the NK cell surface was studied **(**Fig. 2-A**)**. To control for the variability between donors, the percentage of Lamp1$^+$ cells in the control condition was considered as the potential maximum number of degranulating NK cells for each donor, and the percentage of ionomycin-treated degranulating NK cells, compared to the control condition, was calculated **(**Fig. 2-B**)**. After ionomycin treatment, only around 40% (39.72 +/- 18.85) of the potential degranulating NK cells were still able to secrete lytic granules when exposed to target cells, confirming the previous results.

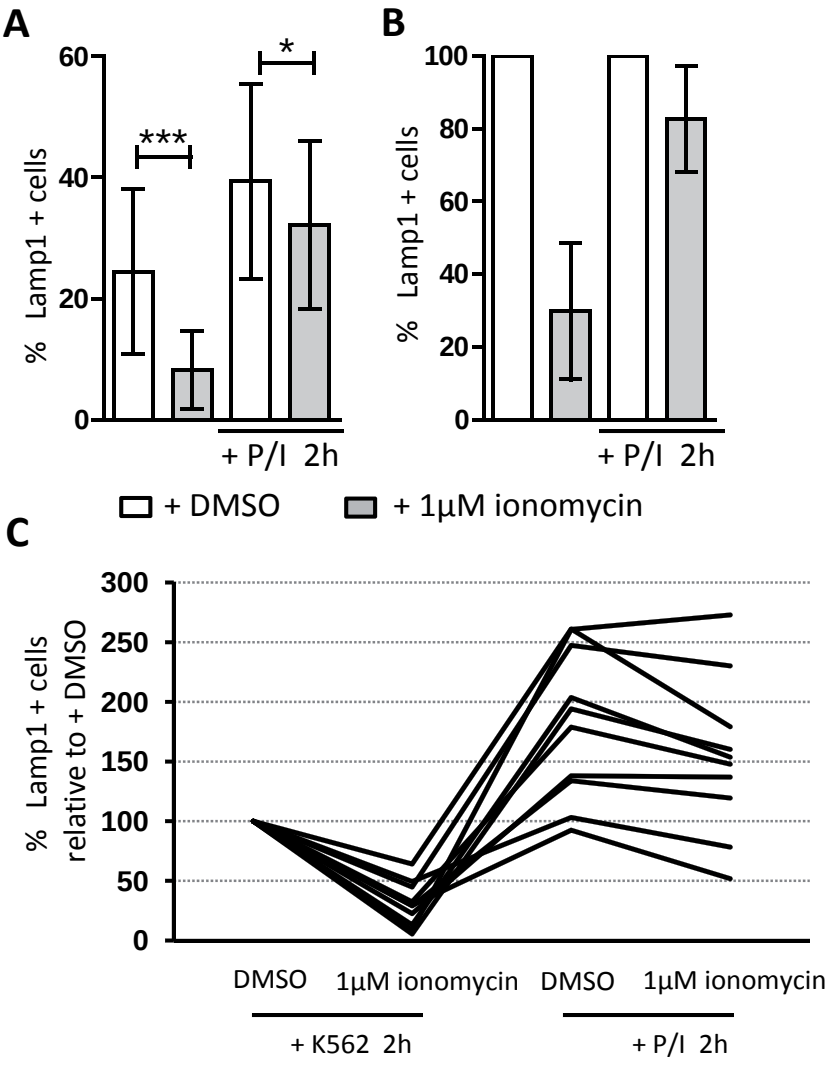


Fig. 2: Ionomycin treated NK cells showed lower ability to degranulate in response to K562, but responded efficiently to PMA /Ionomycin stimulation. (A) Flow cytometry analysis of the percentage of NK cells presenting Lamp1 on surface after coculture with K562 or after PMA/ Ionomcin (P/I) stimulation during 2 hours. **(B** and **C)** Relative numbers of NK cells able to degranulate, compared to control cells, after coculture with K562. **(A** and **B)** Mean data and standard deviation are represented. Two tailed paired Student's t test analysis. *p<0.05, ***p<0.001. (C) Each line corresponds to one experiment. (n=10).

To verify that ionomycin treated cells were still able to secrete their lytic granules, we stimulated ionomycin treated cells with 100ng/mL PMA and 0.5µM ionomycin during 2 hours, in the absence of target cells, and stained for Lamp1 on the NK cell surface. Stimulation by the phorbol ester 12,13-dibutyrate (PMA) and ionomycin stimulates the RasGRP/PKC cascade and induces calcium mobilization, bypassing proximal receptor signaling and driving NK cell activation. Ionomycin treated NK cells were able to respond to these stimuli (82.72% +/- 14.56%). These data indicate that ionomycin treated NK cells contained preformed lytic granules, and that the degranulation machinery downstream PKC and IP_3 was able to signal efficiently and stimulate the degranulation machinery, pointing to defects in either, or both, target cell recognition and in proximal receptor signaling. However the possibility of some defect downstream of PKC and IP_3 could not be discarded from these data since, although the difference between control and ionomycin treated NK cells was low **(**Fig. 2-B**)**, ionomycin treated cells normally didn't reach the level of degranulation of control cells also stimulated with PMA/Ionomycin **(**Fig. 2-C**)**.

This experiment was repeated using a panel of different cell lines as targets (Jurkat, Molt4 and 721.221) and similar levels of dysfunction were observed in each case, demonstrating that ionomycin treated NK cells were unable to degranulate efficiently after stimulation with target cells, independently of the target cells used **(**Fig. 3**)**.

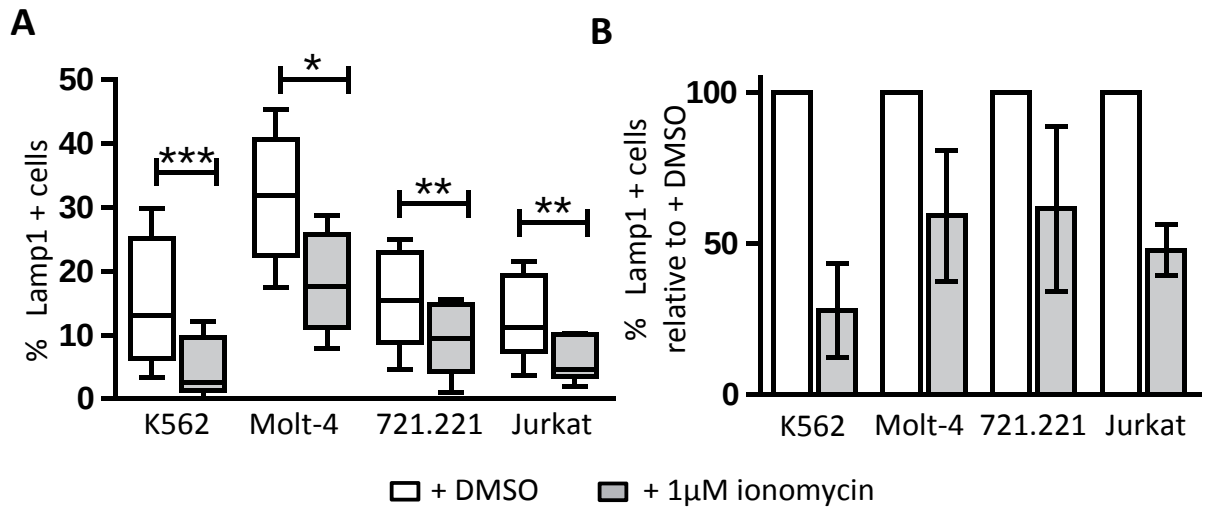


Fig. 3: Ionomycin treated NK cells showed lower ability to degranulate in response to different target cells. (A) Flow cytometry analysis of the percentage of NK cells presenting Lamp1 on surface after coculture with K562, Jurkat, Molt-4 or 721.221 target cells during 2 hours. **(B)** Relative quantities of NK cells able to degranulate compared to control cells. (A and B) Mean data and standard deviation are represented (n=6). Two tailed paired Student's t test analysis was used. *p<0.05, **p<0.01, ***p<0.001.

1.2.2 The effect of ionomycin treatment on NK cell killing

While degranulation is a reasonable measure of NK cell activation it doesn't necessarily correlate with target cell lysis, as killing also depends on multiple other factors such as the adhesion between target and effector cell, the polarized secretion of lytic granules, proper lytic granules content, and the effect of apoptosis-inducing molecules which bind to death receptors on target cells (Fas-Ligand, TNF-α or TRAIL).

To directly analyze target cell lysis, two different target cell lines: K562 and Jurkat cells were used. Both cell lines are sensitive to the effect of lytic granules, but Jurkat cells express Fas and DR4, and so are also sensitive to Fas-Ligand and TRAIL induced death, whereas K562 doesn't express these molecules and so cell death depends exclusively on cytotoxic granule release by NK cells (Zamai et al. 1998).

Target cells were labeled in green with a membrane dye (CFSE or PKH-2), mixed with the NK cells at an E:T ratio of 3:1 and incubated for 1, 3 or 5 hours. As a control for spontaneous cell death, target cells were also incubated alone. At the end of the coculture, cells were stained with Propidium iodide (PI), and analyzed by flow cytometry. The percentage of dead target cells was quantified as the percentage of green cells (CFSE or PKH-2 labeled) that acquired PI. PI is excluded from viable cells as it is membrane impermeable, labeling DNA and RNA of the dead cell population only.

Ionomycin treatment triggered a marked reduction in NK cell killing of both types of target cells **(**Fig. 4**)**. Although ionomycin treatment markedly reduced NK cytotoxicity against both targets, some differences could be observed between K562 and Jurkat cells. In particular, the relative killing of Jurkat cells by ionomycin treated cells was higher than that observed for K562 targets (30.4% +/- 12% of relative to control target cell death after 5 hours of coculture for Jurkat cell as targets, compared to 17.15% +/- 6.107% after 5 hours for K562). This higher relative killing of Jurkat cells compared to K562 by ionomycin treated cells could be due to some level of apoptosis induction by death receptors which could imply that the release of Fas-Ligand or TRAIL is somewhat resistant to ionomycin treatment, but it could also be due to the different sensitivities to NK cell killing of these different target cell lines by the expression of different ligands.

One surprising result from this experiment was the low level of target cell killing by the ionomycin treated NK cells with both target cell lines, even though almost 40% of these NK cells still degranulate after coculture with these target cells **(**Fig. 3**)**. This result could be explained by deficiencies in the content of the cytotoxic granules, in polarized secretion of granules or defects in the adhesion between NK and target cells. These features were analyzed later and are described in **section 2**.

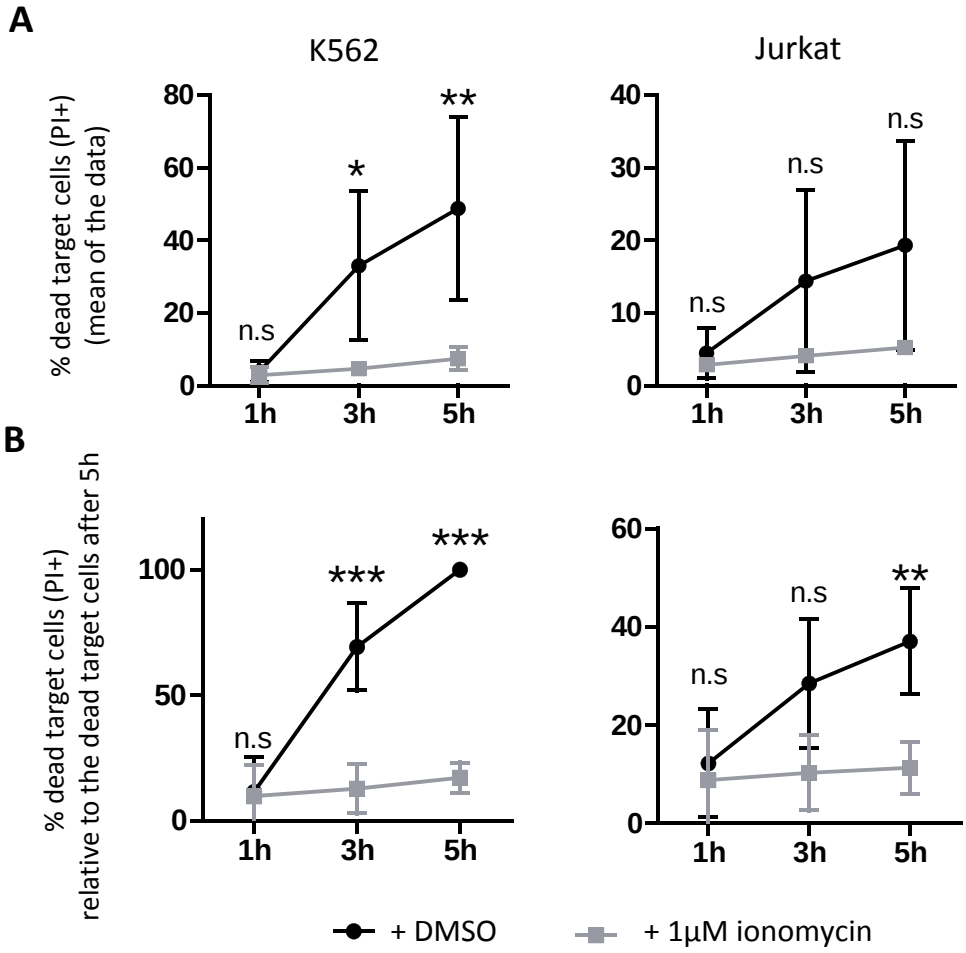


Fig. 4: Ionomycin treated NK cells were unable to kill efficiently K562 or Jurkat target cells. (A) Flow cytometry analysis of the percentage of PI positive K562 or Jurkat cells after coculture with DMSO or 1µM ionomycin treated NK cells during 1h, 3h or 5 hours. **(B)** Percentage of PI positive K562 or Jurkat target cells relative to the percentage of PI positive target cells after 5 hours of coculture with DMSO treated NK cells. **(A** and **B)** Mean data and standard deviation, are represented. (n=4). Two way paired ANOVA analysis and Bonferroni posttest. *p<0.05, **p<0.01, ***p<0.001.

1.2.3 Effect of IL-2 on the degranulation ability of ionomycin treated NK cells

The previous data suggested that NK cells were becoming hyporesponsive after treatment with 1µM ionomycin, as occurs with $CD4^+$ T cells. IL-2 treatment of ionomycin induced anergic CD4+ T cells, rescues the functionality of these cells (Beverly et al. 1992; Macian et al. 2002). In some prior studies hyporesponsive (not ionomycin-induced) NK cells could be rescued by IL-2 stimulation (Abrams and Brahmi 1986; LeFever and Funahashi 1991; Coudert et al. 2005; Tripathy et al. 2008; Sarkar et al. 2013), but not in others (Jewett and Bonavida 1995; Jewett and Bonavida 1996). Thus it was of interest to study the effect of IL-2 on ionomycin treated NK cells.

As shown in figures 5 A and B, when ionomycin treated NK cells were stimulated with 50U/mL of IL-2 during the rest day (after washing to remove the ionomycin) normal degranulation was re-established implying that the ionomycin imposed defect was reversible.

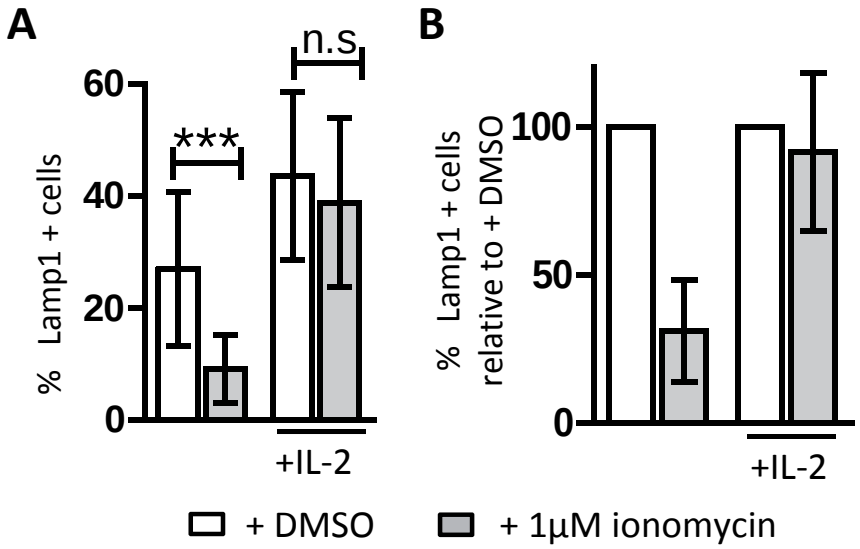


Fig.5: Ionomycin treated NK cells recovered their degranulation ability after being cultured in the presence of IL-2. (A) NK cells were washed and stimulated with 50 U/mL IL-2 during one day, after washing from the ionomycin / DMSO treatment. Cells were cocultured with K562 during 2 hours and stained for Lamp1 expression. **(B)** Relative quantity of NK cells able to degranulate after ionomycin treatment, and IL-2 stimulation, compared to control cells. (n=12). **(A** and **B)** Mean data and standard deviation are represented. Two tailed paired Student's t test analysis. ***p<0.001.

1.2.4 Effect of IL-12 / IL-18 and IFN type 1 on degranulation by ionomycin treated NK cells

Inflammatory cytokines have been proposed to be the third signal needed for T cell activation (after TCR and costimulatory signals), and for example IL-12 is known to rescue exhausted $CD8^+$ T cells (Schurich et al. 2013). IL-12, IL-18 and type 1 interferons have also been used to recover hypofunctional NK cells (Abrams and Brahmi 1986; Yokoyama and Kim 2006; Tripathy et al. 2008; Mazumdar et al. 2013). Type 1 interferons and IL-12/ IL-18 are cytokines secreted by different cell types including activated macrophages, dendritic cells or neutrophils, and are known to directly stimulate NK cell activity (Trinchieri 1995; Swann et al. 2007; Chaix et al. 2008; Martinez et al. 2008). In the context of inflammation these cytokines will be secreted and can act as potent activators of NK cells function.

To study the influence of these cytokines on ionomycin treated NK cells, we stimulated them during the resting day with a combination of 10ng/mL IL-12 plus 10ng/mL IL-18, or with 100 and 1000U/mL of IFN-α in combination or not with 10ng/mL IL-18.

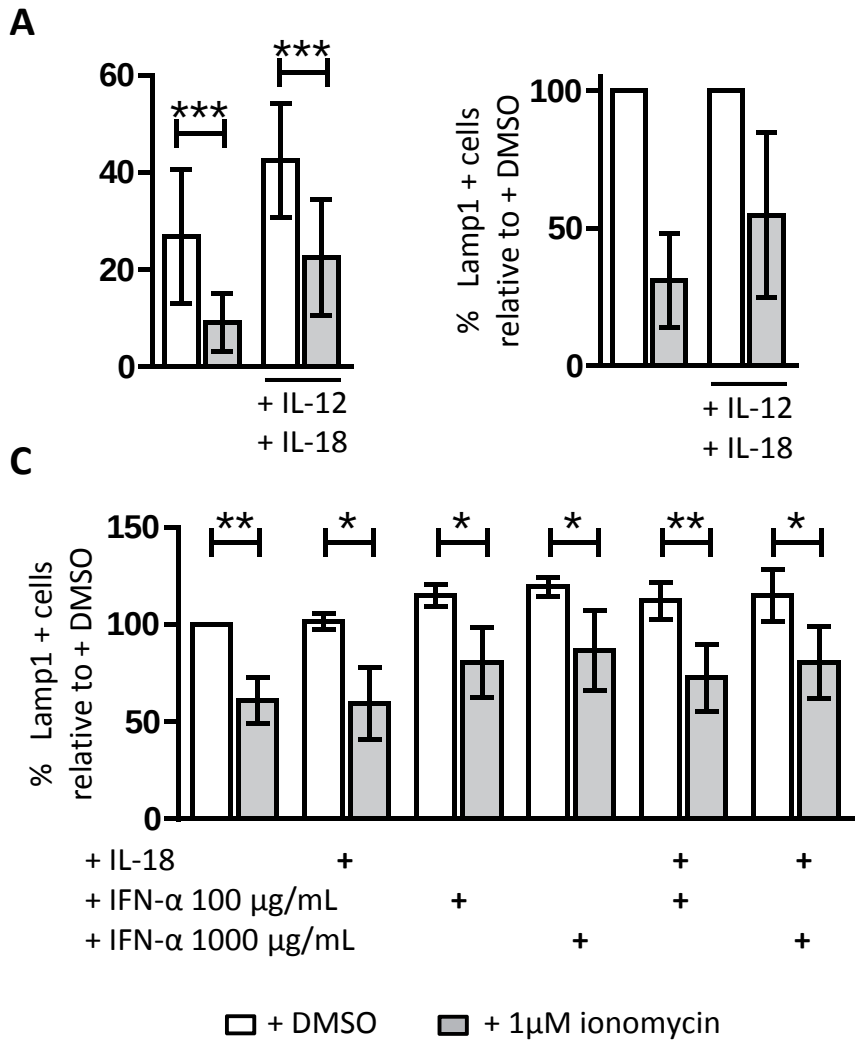


Fig. 6: Ionomycin treated NK cells did not recover their functionality after exposure to IL-12 / IL-18 or to IFN-α. NK cells were stimulated with **(A** and **B)** IL-12 / IL-18 **(C) o**r IFN-α +/- IL-18, during the freshly isolated day, and stained with Lamp1 antibodies after 2 hours coculture with K562. **(B** and **C)** Relative quantity of NK cells able to degranulate compared to control condition (DMSO). **(A** and **B** n=14; **C** n= 3**) (A, B** and **C)** Mean data and standard deviation are represented. **(A)** Two tailed paired Student's t test analysis was used. ***p<0.001. **(C)** Paired two way ANOVA analysis and Bonferroni post-test. *p<0.05, **p<0.01.

Although both stimuli, IL-12 + IL-18 and IFN-α were able to increase the number of NK cells degranulating, the difference between control and ionomycin treated cells was maintained **(**Fig.6**).** Therefore, stimulation with these cytokines does not restore the functionality of ionomycin-treated NK cells.

1.3 Cytokine production by ionomycin treated NK cells

Another important role of NK cells is the production of cytokines and chemokines that can regulate the function of other immune cells. One of the main cytokines secreted by NK cells is IFN-γ (Fauriat et al. 2010). It has been reported that freshly isolated $CD56^{dim}$ NK cells contain preformed IFN-γ mRNA and so are able to produce and secrete IFN-γ very quickly (De Maria et al. 2010). However, when NK cells were cocultured with K562 target cells during 6 hours in the presence of monensin, which impairs protein trafficking and leads to the accumulation of IFN-γ in the Golgi apparatus, IFN-γ production by NK cells

was not detected by intracellular flow cytometry, perhaps due to the lower sensitivity of the flow cytometry technique.

Therefore to increase the production of IFN-γ, control and ionomycin treated NK cells were stimulated with IL-12 and IL-18 (10ng/mL of each) during the resting day. This is a stimulus that NK cells can receive from macrophages and dendritic cells and enhances IFN-γ production by NK cells (Chaix et al. 2008). When stimulated with IL-12/IL-18, hyporesponsive NK cells produced much less IFN-γ than untreated NK cells and while the production of IFN-γ by control NK cells was further increased on exposure to target cells, this was not observed for ionomycin treated NK cells. When stimulated with IL-2 both control and ionomycin treated cells were able to produce similar amounts of IFN-γ when exposed to target cells **(**Fig. 7**)**, implying that the changes suffered by ionomycin-treated NK cells were reversible. We also stimulated control and ionomycin treated cells with 100ng/mL PMA and 0.5µM ionomycin during 6 hours, in the absence of target cells, and observed no difference between the percentage of IFN-γ positive control and ionomycin treated cells, however, the median fluorescence of the ionomycin treated IFN-γ positive cells was lower (62.41 % +/- 31.55 of the median fluorescence intensity seen for the control cells treated with PMA/Ionomycin) (data not shown), suggesting that each cell made less of this cytokine.

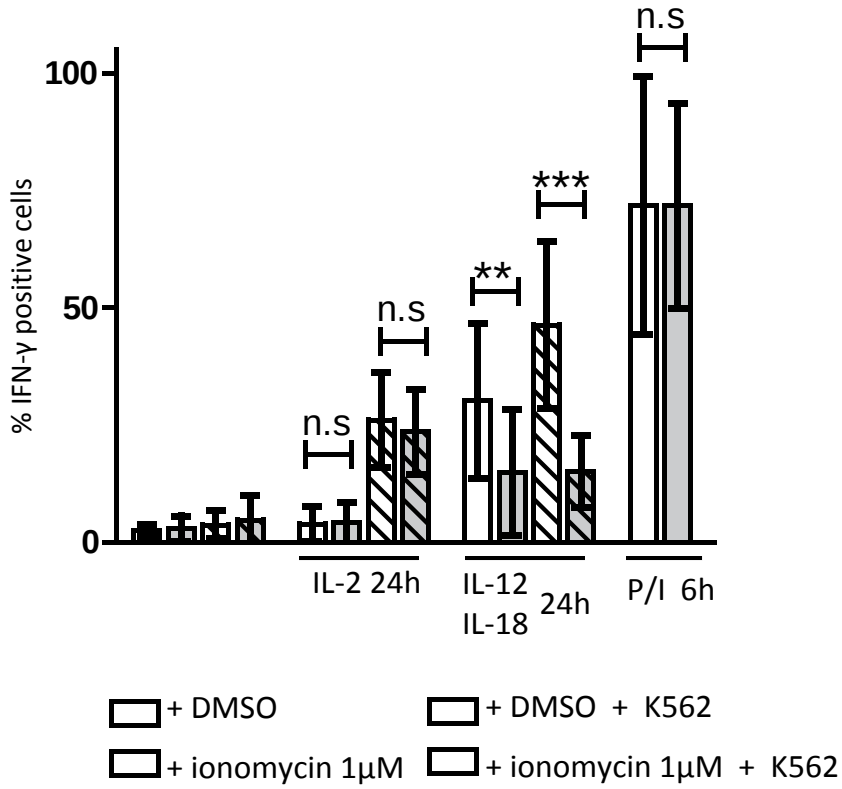


Fig. 7: Ionomycin treated NK cells were unable to secrete IFN-γ as efficiently as control cells after IL-12 / IL-18 stimulation, but they were effective producers when stimulated with PMA/Ionomycin or after overnight stimulation with IL-2. IFN-γ production by NK cells was analyzed by flow cytometry. For all stimulation conditions control (white bars), ionomycin treated (grey), control cocultured with K562 for 6h (lined white) or ionomycin treated cocultured with K562 for 6 hours (lined grey) where analyzed. Cells were unstimulated, stimulated with IL-2 overnight, stimulated with IL-12/IL-18 overnight or with PMA/Ionomycin during 6 hours. Mean data and standard deviations are represented (n=6). Two tailed paired Student's t test analysis. *p<0.05, **p<0.01, ***p<0.001.

The low levels of IFN-γ induced by target cells on ionomycin treated NK cells might be explained by failures of target cell recognition, adhesion or stimulation, which are upstream of PKC and IP3 stimulation. However, the lower levels of IFN-γ production observed when the cells were stimulated with IL-12 and IL-18 in the absence of target cells implied signaling defects or lack of proper cytokine recognition. A lower stability of IFN-γ mRNA, or epigenetic changes around to the IFN-γ gene may also explain these defects. Some of these possibilities were analyzed in **section 3**.

1.3.1 Autoregulation

NK cells are known to be able to secrete a range of immunoregulatory cytokines including IL-10, IL-13 or TGF-ß. Thus a possible explanation for the defects observed in ionomycin treated cells, was that they were secreting molecules which acted to inhibit their capacity to mediate cytotoxicity or secrete pro-inflammatory cytokines.

To test this hypothesis, PKH-2 stained NK cells maintained with IL-2 were cocultured with either vehicle control or ionomycin (1µM) treated NK cells, for 24 hours in the absence of IL-2. After the coculture, the degranulation ability of activated NK cells (PKH-2 stained) as well as the control and ionomycin treated NK cells (PKH-2 negative) was analyzed.

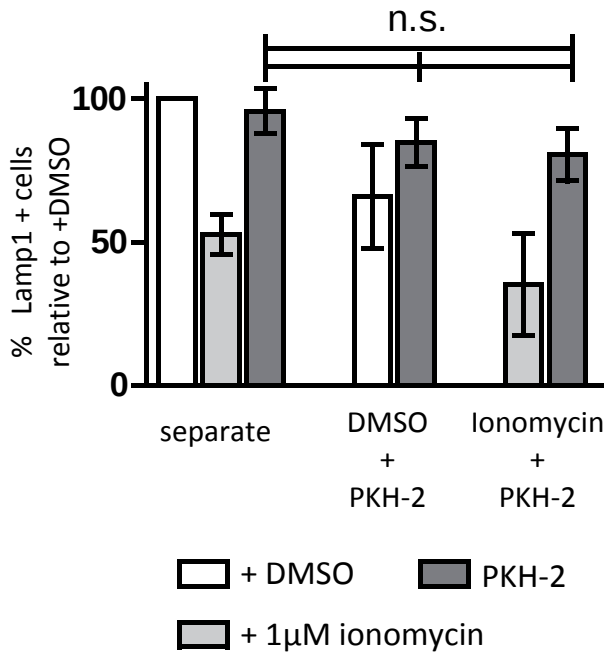


Fig. 8: Ionomycin treated NK cells were not secreting any immunosuppresive factor. NK cells were treated with 1µM ionomycin (light grey) or DMSO (white) during 16 hours. After washing, PKH-2 stained NK cells from the IL-2 cultures were added and cocultured with DMSO or ionomycin treated NK cells for 24 hours (dark grey). The percentage of NK cells presenting Lamp1 on surface after coculture with K562 was evaluated by flow cytometry. Data represent the relative percentage of Lamp1$^+$ cells, related to the DMSO alone condition. Mean data and standard deviations are represented (n=4). Paired one way Anova analysis and Tukey´s multiple comparison test.

No differences in the ability of the PKH-2 labeled NK cells to degranulate on exposure to target cells was observed after coculture with either DMSO or ionomycin treated NK cells **(**Fig. 8**)**. These observations strongly suggest that ionomycin treated NK cells do not release any immunosuppressive factors into the culture media and suggest that the defect is intrinsinc to the hyporesponsive NK cell.

1.4 Effect of ionomycin treatment on NK cell viability

Although ionomycin treated NK cells were functional as they responded to PMA/Ionomycin stimulation as efficiently as control cells and culture in IL-2 rescued the hyporesponsive phenotype, it was important to analyze the viability of these cells to discard the possibility that the $Lamp1^+$ population observed in ionomycin treated cells **(**Fig.1-A**)** was due to cells which enter into apoptosis after the ionomycin treatment.

To analyze NK cell viability we cocultured NK cells with K562 for 2 hours, and stained them with CD56 (to distinguish between NK cells and target cells), Lamp1 (to define degranulating and non-degranulating NK cells) and Annexin-V /7-AAD (7-Aminoactinomycin D) to analyze the viability of degranulating and non-degranulating NK cells in the control and ionomycin treated NK cells. Annexin-V stains phosphatidylserine which appears on the surface of non-permeabilised cells in early apoptosis states due to the loss of the normal asymmetric distribution of phosphatidylserine. 7-AAD, similar to propidium iodide, has high affinity for DNA and intercalates with it, but since it is cell impermeant, 7-AAD only stains cells with disrupted membranes, such as cells in late apoptosis states, or necrotic cells.

These experiments demonstrated that the ionomycin treated NK cells were as viable as control cells, although the few ionomycin treated $Lamp1^+$ NK cells stained slightly more Annexin V positive than control cells **(**Fig.9**)**. It was surprising to find this high level of phosphatidylserine positive cells in the $Lamp1^+$ population in both control and ionomycin treated cells. However, this staining was probably not due to NK cell apoptosis, but rather to the process of degranulation, as this extracellular Annexin-V staining has also been described in mast cell degranulation processes (Demo et al. 1999), and different members of the Annexin family have been described to have a role in membrane fusion processes, including degranulation (Gerke et al. 2005). Thus phosphatidylserine expression at the cell surface would be a consequence of the degranulation process similar to the increased exposure of Lamp1 at the cell surface.

These data confirmed the viability of the ionomycin treated cells; and the observed co-staining of Lamp1 and Annexin-V strongly suggests that ionomycin treated cells were able to exert real degranulation processes.

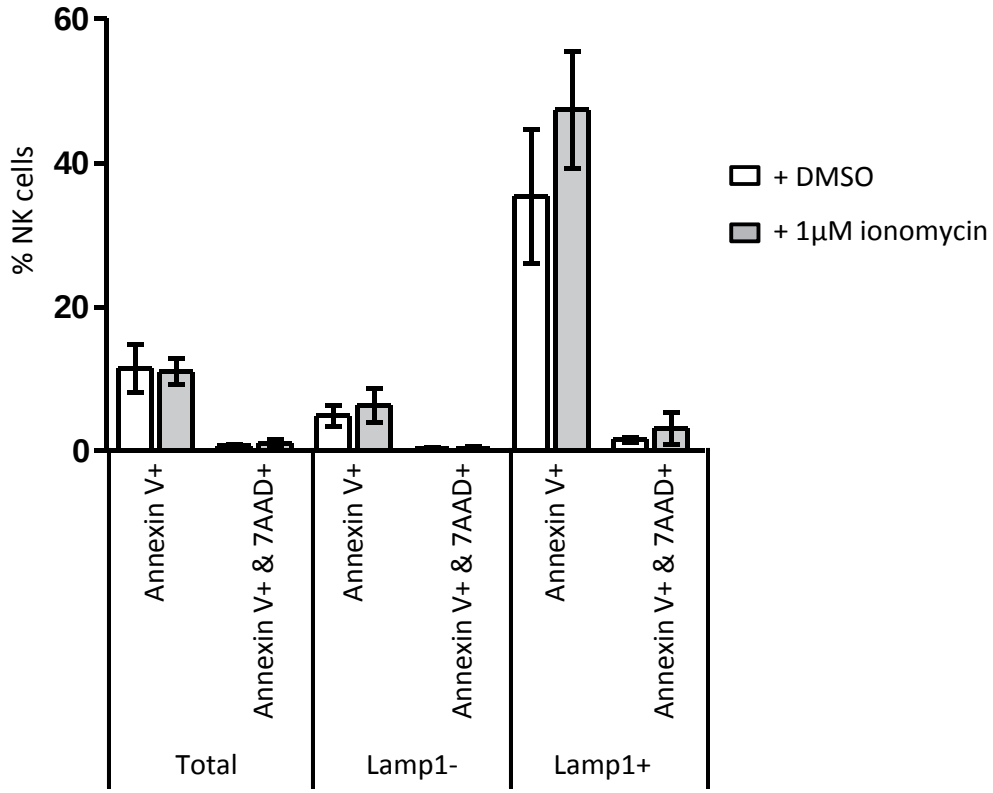


Fig. 9: Ionomycin treated NK cells were as viable as control cells. The Lamp1^{+} population was more Annexin-V positive than the Lamp 1^{-} in control and treated cells. Control and ionomycin treated NK cells were stained with Lamp1*APC, Annexin-V*FITC and 7-AAD after coculture for 2 hours with K562 target cells. Mean data and standard deviations are represented (n=3). Two tailed paired Student's t test analysis was used.*$p<0.05$.

2. Cytotoxic recognition by ionomycin treated NK cells

The previous work characterizing the responsiveness of ionomycin treated NK cells, had shown that they were ineffective killers **(**Fig. 3 and 4**),** and unable to produce IFN-γ after IL-12 / IL-18 stimulation and coculture with target cells **(**Fig. 7**)**. These data established the existence of defects on target cell recognition, but left open the stage(s) of the killing process in which ionomycin treated NK cells were defective.

2.1 Conjugate formation

After the very initial stages of NK cell/target cell contact and recognition, the interaction between cells has to be stabilized. This process requires the specific interaction of adhesion molecules such as integrins that collaborate in initial cell to cell contacts, and later when the cell is selected as target, produce strong binding following activation of these integrins by inside-out signaling.

The ability of NK cells to form tightly bound conjugates with target cells was studied using a flow cytometry based protocol where NK cells and target cells were labeled with different fluorescent dyes (PKH-2 - green, and PKH-26 - red were used) and co-incubated at 37°C. Samples were collected after 2, 5, 10, 20, 30, 45, and 60 minutes and vortexed to disrupt loosely associated cells and fixed. After collecting all the time points, the double positive events which represent NK cells firmly adhered to target cells **(**Fig. 10-A**)** were quantified by flow cytometry.

Ionomycin treated NK cells were less able to form conjugates, with the maximum differences observed between 20 and 45 minutes **(**Fig. 10-B**)**. Due to high variation between NK cells from different donors, the data are presented as the percentage of ionomycin treated NK cells forming conjugates with K562 relative to the percentage of DMSO treated NK cells (control cells) forming conjugates with K562 at the same time points **(**Fig. 10-C**)**. In this analysis all the time points except the 60 minutes point were <1 (control cells level), and represent between a 44% (at 2 minutes) and a 26% (45 minutes) reduction in the percentage of ionomycin treated cells that form conjugates compared to the formation of conjugates by control NK cells.

This difference in the percentage of cells forming conjugates could be due to the existence of a lower percentage of NK cells able to form stable conjugates with targets, or to the formation of less stable conjugates. Less stable conjugates could be explained by defects in target cell recognition, by differential expression of NK cell activating or inhibitory receptors; or to an inability to form stable interactions with target cells, by for example lower levels of adhesion molecules.

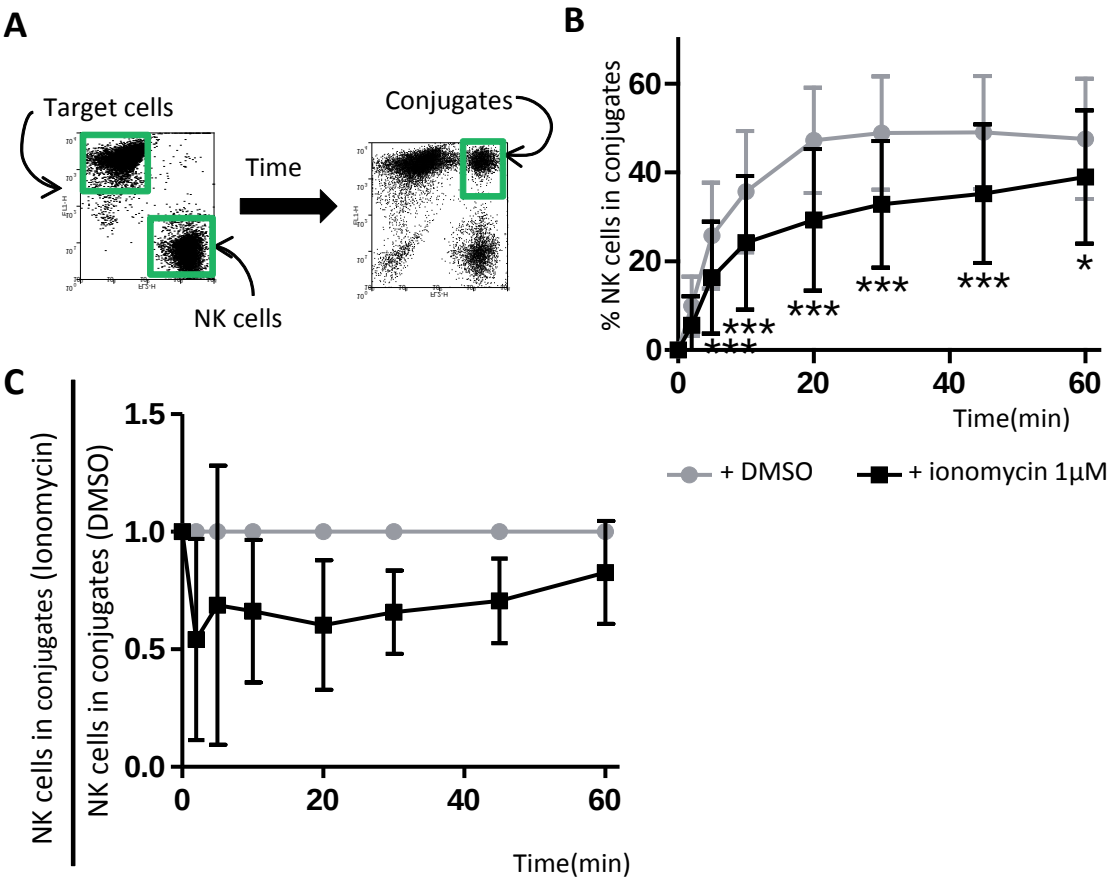


Fig. 10: Ionomycin treated NK cells formed conjugates less efficiently. Conjugate formation was analyzed by flow cytometry. (A) NK cells and target cells were stained with different dyes. Conjugates appear as double positive events. **(B) T**he percentage of NK cells forming conjugates varies over time. Mean data are represented. **(C)** The number of ionomycin treated NK cells forming conjugates is expressed relative to the percentage of NK cells forming conjugates in the control condition. Mean data and standard deviations are represented. (n=15). Paired two way ANOVA analysis and Bonferroni posttest.*p<0.05, ***p<0.001.

However, ionomycin treated NK cells were still able to form a significant amount of conjugates with target cells, and the difference between ionomycin treated and control cells was just 15% after 60 minutes of coculture with K562 (ionomycin treated cells forming conjugates referred to the cells able to form conjugates in the control condition Fig.10-C). This small difference didn't correlate with the much lower levels of target cell killing observed (17.154% +/- 6.107% after 5 hours Fig. 4). For this reason, although a lower efficiency of conjugate formation could be part of the explanation of the deficient target cell killing, more steps of the NK cell killing process were analyzed.

2.2 LFA-1 and actin polarization

One of the first steps that take place after NK recognition of a target cell is the polarization of adhesion molecules as LFA-1 and Mac-1 into the pSMAC where they contribute to the formation of conjugates between the NK and the target cell. Integrins are then further activated by inside-out signals provided by the activating stimulus. In contrast to TCR dependent LFA-1 activation in T cells, in NK cells, LFA-1 is sufficient to start its own inside-out signals (Barber and Long 2003). This activated LFA-1 will also start signaling cascades, and contribute to granule polarization and actin polymerization (Barber et al. 2004). Rearrangements of the actin cytoskeleton lead to the accumulation of filamentous actin (F-actin) and the formation of a talin/actin/LFA-1 pSMAC collar which stabilizes the union between effector and target cells, generates the platform for a localized delivery of cytotoxic granules, aids the establishment of new signaling complexes and facilitates the accumulation of lipid rafts to the synapse (Vyas et al. 2002). Actin polymerization also favours integrin polarization towards the pSMAC (Orange et al. 2003), and disruption of actin polymerization processes results in severe impairment of NK cell activity (Orange et al. 2002).

To assess if ionomycin treated cells were able to efficiently polarize molecules towards the immune synapse the behaviour of LFA-1 and F-actin was studied due to the important roles of these molecules both in the formation of stable synapses and in the further transmission of signals.

Immune synapse formation was analyzed by confocal microscopy. K562 target cells were stained with CMAC dye to distinguish them from effector cells. Then NK cells and CMAC labeled K562 cells were cocultured over poly-L-lysine coated coverslips for one hour, before the samples were fixed and stained with LFA-1-specific monoclonal antibody and Phalloidin, a fungal toxin which specifically binds F-actin. Samples were visualized by confocal microscopy and conjugates were selected for image collection. Later the ratio of LFA-1 and Phalloidin fluorescence between the synapse, and non synapse membrane zones of control and ionomycin treated NK cells was quantified using ImageJ-FIJI (Schindelin et al. 2012). Briefly, the signals in the LFA-1 channel of the background, membrane in the synapse-zone, and non synapse-zone, were measured by drawing areas with the FluorescenceRatio plugin for ImageJ (created by C.O.Sánchez-Sorzano) which calculated the fluorescence ratio from the intensity of the signals in the synapse *vs.* non synapse membrane regions. For quantifying F-actin polarization, the Synapse measures plugin (Calabia-Linares et al. 2011) (which also measures the target cell F-actin contribution to the immune synapse) **(**Fig. 11-A**)** was used.

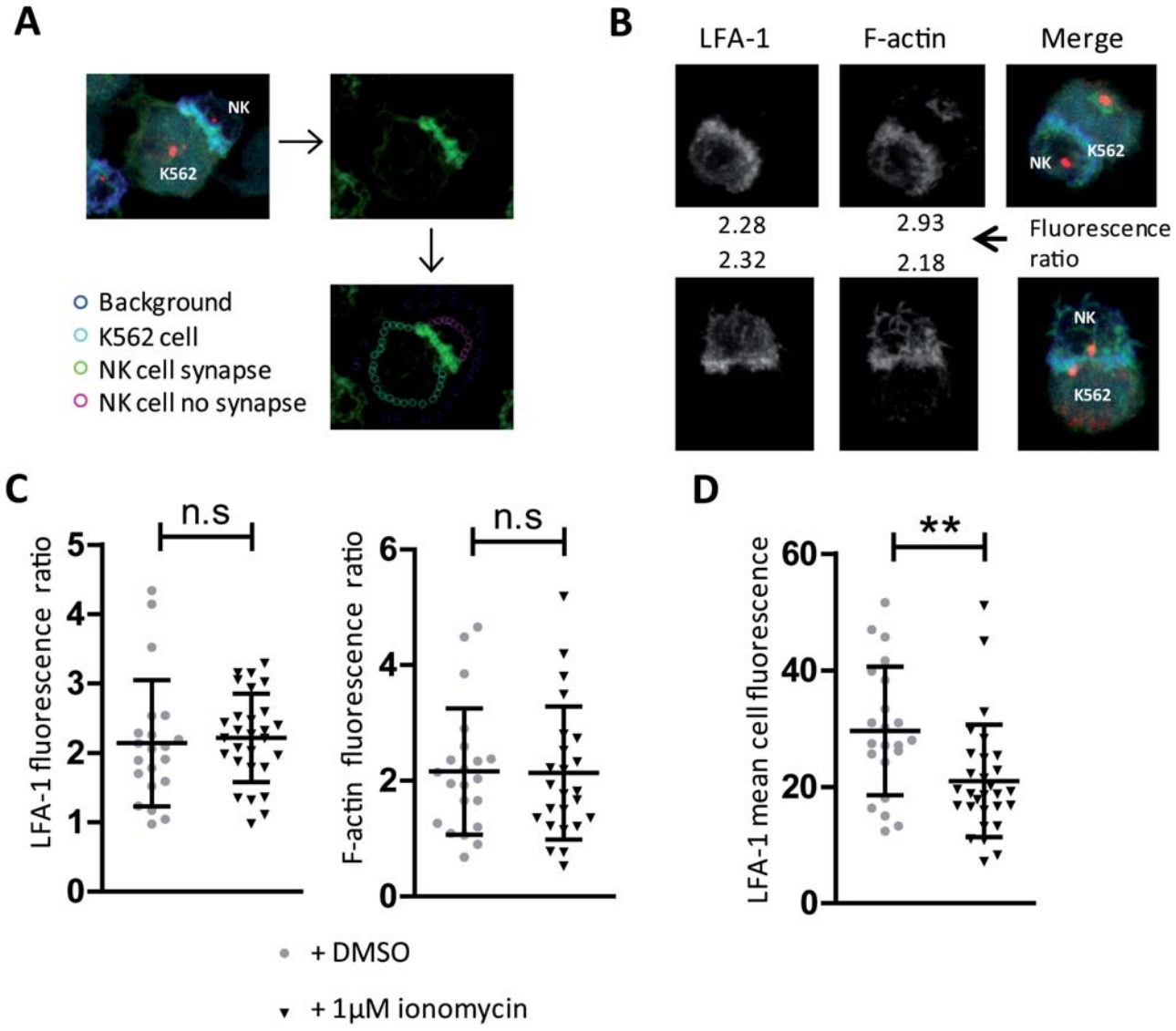


Fig. 11: Ionomycin treated NK cells were able to polarize LFA-1 and accumulate F-actin in the immune synapse with target cells as efficiently as control cells. NK cells were allowed to interact with CMAC labeled K562 cells (light blue) and to attach to poly-L-lysine coated coverslips during 1hour. Cells were fixed and stained with primary and secondary antibodies: anti-LFA-1 antibody (H1III clone) & GAM-Alexa 648 (dark blue), anti-pericentrin antibody (Ab4488) & GAM-Alexa 546 (Red); and with Phalloidin– Alexa 488 (green). **(A)** LFA-1 and F-actin polarization to the NK immune synapse was quantified using ImageJ plugins Fluorescence ratio (LFA-1) and Synapse measures (F-actin). Representative example of F-actin polarization quantification. **(B)** Representative examples of control (top) and ionomycin treated (lower) cells staining of single Alexa 648 (LFA-1) and Alexa 488 (F-actin) channel colors, and their merge. **(C)** Quantification of LFA-1 and F-actin fluorescence ratios. Each line corresponds to one cell analyzed. **(D)** Mean fluorescence intensity of LFA-1 – Alexa 648 staining in control and ionomycin treated cells was measured by ImageJ. Mean data and standard deviations are represented. Each symbol corresponds to one cell analyzed. (n=22 DMSO, n=29 Ionomycin) Mann Whitney Test. **p<0.05.

In general, ionomycin treated NK cells expressed lower amounts of LFA-1 compared to control cells. To quantify LFA-1 staining the mean fluorescence of each cell was measured using ImageJ and these data confirmed our observation **(**Fig. 11-D**)**, however some ionomycin treated NK cells that expressed high amounts of LFA-1 were also detected **(**Fig. 11-A2**)**. Interestingly, despite the lower levels of LFA-1 expression found in ionomycin treated NK cells, those NK cells that formed conjugates with K562 target cells were able to polarize LFA-1 with the same efficiency, and accumulate F-actin in the immune synapse zone **(**Fig. 11-B, C**)**. Further analyses of the decreased LFA-1 expression are described in **section 3**.

2.3 Cytotoxic granule polarization

After the formation of stable conjugates between the NK and the target cell, receptors and adhesion molecules polarize to the cytotoxic immune synapse accompanied by the creation of an F-actin ring. The newly active signaling cascades will lead to cytotoxic granule polarization, driven by the MTOC (microtubule organizing centre), to the immune synapse (Orange 2008).

The polarization of cytotoxic granules was analyzed by confocal microscopy. As before, K562 target cells were stained with CMAC and cocultured with control and ionomycin treated NK cells on poly-L-lysine coated coverslips for one hour. The samples were then fixed and stained with a perforin-specific antibody to label cytotoxic granules. Finally, the number of NK cells forming conjugates with their cytotoxic granules polarized towards the site of contact between the NK cell and the target cell was quantified. In these experiments three different types of conjugates were counted depending if the lytic granules were non polarized, fully-polarized and partial polarized (when most of the granules are polarized but there are granules also in other zones of the cell) **(**Fig. 12-A**)**.

As expected from the previous flow cytometry analysis **(**Fig.10**)** in these experiments the ionomycin treated NK cells formed lower numbers of conjugates with the target cells. A small tendency for ionomycin treated NK cells to have less conjugates with their granules polarized was observed, however, many of those ionomycin treated NK cells which formed conjugates, were able to polarize their granules efficiently **(**Fig.12-B**)**.

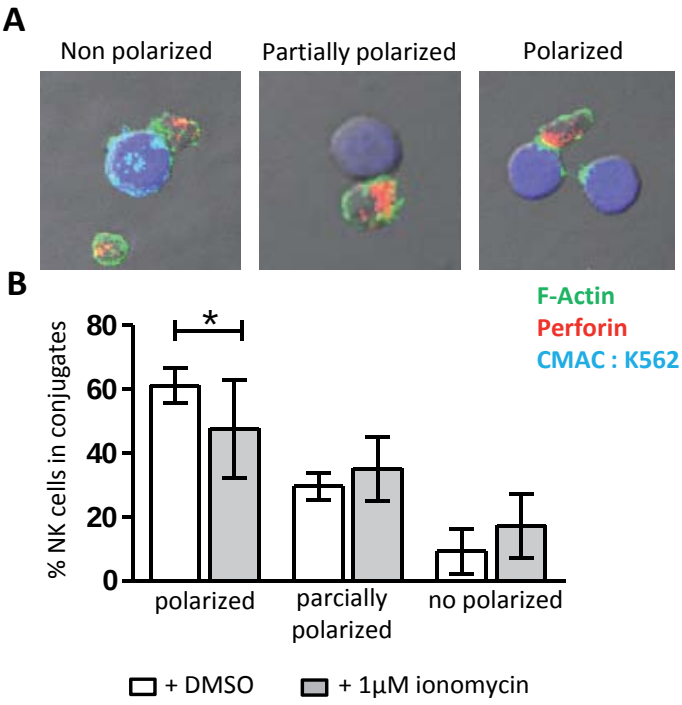


Fig. 12: Ionomycin treated NK cells forming conjugates with target cells, polarized lytic granules as efficiently as control cells. (A) Three different granule polarization situations were defined: non polarized, partially polarized and polarized. F-actin (Phalloidin-Alexa 488) appears in green, granules in red (Perforin antibody + GAM-Alexa 568) and target cells in blue (CMAC dye. **(B)** The state of lytic granules polarization was quantified among the conjugates formed between NK cells and K562 cells by confocal microscopy. Mean data and standard deviations are represented. Paried two way ANOVA analysis and Bonferroni posttest. *p<0.05

3. Characterization of receptors expressed by ionomycin treated NK cells

The data outlined in **chapters 1** and **2** indicated that ionomycin treated NK cells were unable to kill target cells or respond to treatment with the cytokines IL-12 and IL-18 efficiently. Analysis of specific steps of the killing process showed defects in conjugate formation with target cells, and lower levels of LFA-1 expression. However many of those ionomycin treated NK cells that were able to form conjugates, were competent to polarize LFA-1 and their lytic granules towards the cytotoxic immune synapse.

The NK cell population of an individual consists of cells expressing different inhibitory receptors in a stochastic manner. NK cells responsiveness is thought to be established during their development and is influenced by the repertoire of receptors they express and the levels of expression of these molecules. According to this model (the rheostat model) the NK subset of each individual is composed of a heterogeneous population of NK cells expressing variable repertoires of activating and inhibitory receptors, covering a spectrum of responsiveness and adapted to the individual (Brodin et al. 2009; Shafi et al. 2011). This variation in the threshold required to trigger a response between circulating NK cell clones of a given individual could be another explanation of why around 40% of the potential degranulating NK cells were still able to secrete lytic granules after treatment with ionomycin **(**Fig. 2**)**. If ionomycin treatment acts by "tuning" the NK cell activation threshold, then although after treatment many cells would be unable to reach their activation frontier (rendered unresponsive), it would not be surprising if there also existed some cells still able to respond to target cell stimulation, form stable conjugates, and able to polarize LFA-1, F-actin and lytic granules **(**Fig. 11 and 12**)**. This hypothesis would also explain why ionomycin-exposed NK cells still responded to treatment with a very powerful stimulus such as the combination of PMA and ionomycin. Given that it is a balance of signals coming from activating and inhibitory receptors that determines whether NK cells reach the threshold signalling required for activation, NK cells were stained for different activating and inhibitory receptors. Lower levels of activating receptors or adhesion molecules, and/or higher levels of inhibitory receptors could explain the absence of target cell recognition and less stable conjugate formation. To complement these data, experiments of redirected-antibody dependent cell cytotoxicity and analysis of calcium flux were performed to study the functionality of individual receptors or combinations of different molecules. Finally, the expression of molecules that are differentially expressed between different populations of NK cells was also examined to test whether ionomycin treatment preferentially affected, or spared, the function of particular NK cell subpopulations.

3.1 Activating and inhibitory receptors

NK cells express a large variety of different activating receptors. As described in the introduction, various articles have described different changes in the phenotype of hyporesponsive NK cells. However, to date, no clear relationship has been established between these changes and the hyporesponsive state.

Flow cytometry was used to analyze the expression of different NK activating and inhibitory receptors by DMSO and 1µM ionomycin treated cells but no consistent difference was observed between these populations. The activating receptors analyzed were: NKG2D, NKp46, NKp30, CD16, CD94, 2B4 and CD28. The different inhibitory receptors studied were: KIR2DL1 (CD158a), KIR2DL2/DL3 (CD158b), KIR3DL1 (CD158e1); CD94, LILRB1 (ILT2, CD85j) and CD161 (KLRB1).

The induced expression of molecules as PD-1 and CTLA-4 has been related to T cell anergy and exhaustion (Greenwald et al. 2001; Crawford and Wherry 2007) and so the effect of ionomcyin treatment on NK cell expression of these molecules was also studied. In a few donors a small PD1 positive population could be detected after ionomycin treatment, or a slight movement of the whole the cell population was noted, but this was not consistent between different donors **(**Fig. 13-A**)**. No induction of CTLA-4 was observed.

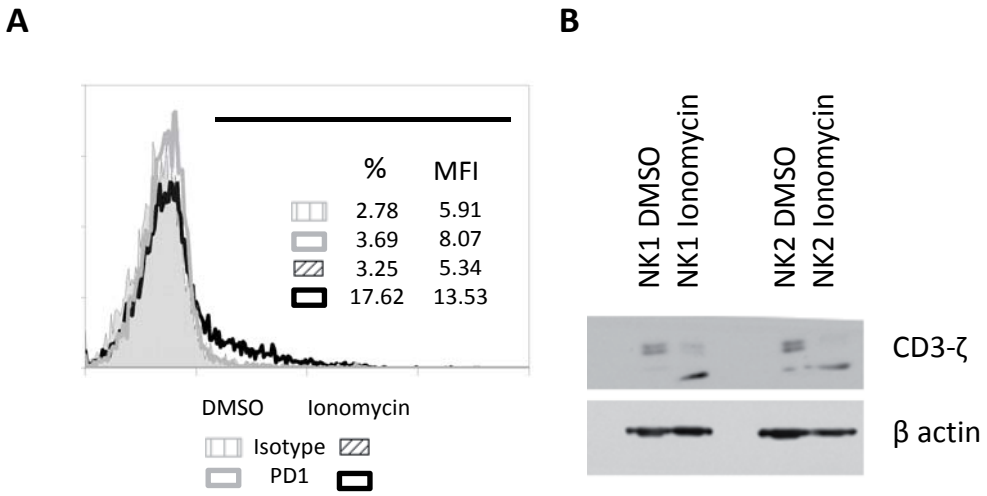


Fig. 13: Ionomycin treated NK cells expressed the same level of activating and inhibitory receptors. In some cells lines small increases in PD1 expression could be observed but this was not consistent between different cell lines. CD3-ζ chain expression is decreased in ionomycin treated cells. (A) Representative histogram of flow cytometry analysis of DMSO and ionomycin treated cells stained with isotype control and PD1-specific antibodies. Numbers represent the percentage of positive cells (percentage of cells under the bar), and the whole population MFI. **(B)** Representative western blot analysis of 50µg of total cell lysates using NP40 lysis buffer. NK cells from two different donors, treated and untreated with ionomycin are shown. Membranes were exposed to CD3-ζ and ß-actin antibodies.

The CD3-ζ chain is an adaptor molecule used by various NK cell activating receptors (CD16, NKp46 and NKp30) for signal transmission. Downregulation of this molecule has been reported in tumor associated NK cells (Lai et al. 1996; Patankar et al. 2005), and in mouse NK cells chronically exposed to NKG2D ligand expressing target cells (Coudert et al. 2005; Coudert et al. 2008; Hanaoka et al. 2010), further loss of CD3-ζ has also been related with CD4+ T cell anergy (Heissmeyer et al. 2004). Western blot analysis showed decreased expression of this molecule in ionomycin treated NK cells **(**Fig. 13-B**)** pointing to defects in activating receptor signal transmission.

3.2 Adhesion molecules

In pevious experiments of confocal microscopy lower levels of expression of LFA-1 (αLß2 integrin, CD11a:CD18) in the 1μM ionomycin treated cells were noted **(**Fig. 11-D**)**. Integrins are known to switch from a compact or closed conformation to an open active conformation (Luo et al. 2007) changing their avidity (regulated by receptor clustering), and affinity for their ligand (changes in integrin conformation, with three steps: closed, intermediate and open or high affinity). Thus, to confirm the previous observation by confocal microscopy of the lower expression of the αL subunit, it was analyzed by flow cytometry. Other integrin molecules as the αM subunit (CD11b) and the ß2 subunit (CD18) were also analyzed since both αL and αM associate with ß2 to arrive to the cell surface. A range of mAbs were used to study αL subunit expression. The HI111 antibody, which binds the I-domain of the αL chain and binds all forms of LFA-1 although it preferentially recognizes LFA-1 in its closed conformation, since it binds with lower affinity to LFA-1 locked in the open conformation or activated by divalent cations (Ma et al. 2002). To study open states of LFA-1 the expression of αL was analyzed with an antibody (NKI-L16) specific for the active conformation of integrins (Keizer et al. 1988). This mAb recognizes an epitope exposed only in αL integrins in a Ca2+-bound, extended conformation (van Kooyk et al. 1991), and reactivity with this mAb has been related to clustering-dependent increases in the avidity of αL integrins for their ligands. Reactivity with the ab24 antibody which recognizes an epitope of the β2 chain I-like domain which is accessible to the antibody only in the Mg2+-dependent extended or high affinity conformation of LFA-1 (Stewart et al. 1996), and one antibody specific for the active conformation of αM (cbrm1/5) (Diamond and Springer 1993) was also examined, but no cell staining was observed with these mAbs **(**Fig. 14-A**)**.

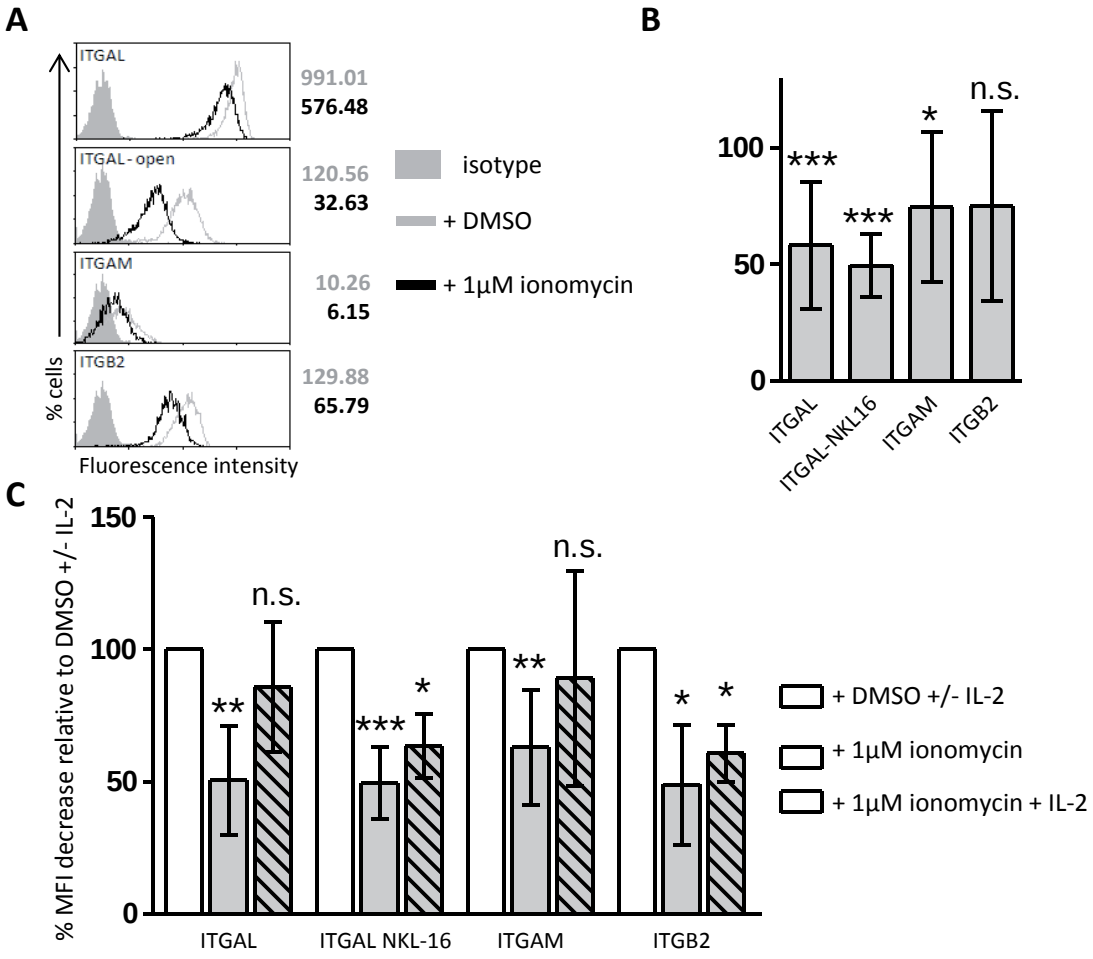


Fig. 14: Ionomycin treated NK expressed lower levels of molecules of the integrin family. (A) Representative histograms of closed αL, active αL, αM and ß2 expression by flow cytometry. Isotype control: grey filled. Control cells: grey line. Ionomycin treated cells: black line. **(B) Relative decrease of the MFI expression of the different integrin molecules relative to the expression of co**ntrol cells (DMSO) (n=14 αL, n=10 αL NKL-L16, n=13 αM, n=12 ß2). **(C)** Relative decrease of the MFI expression of the different integrin molecules relative to the expression of control cells (DMSO), only considering those that suffered a decrease in expression. Gray bars: cells without IL-2 stimulation (n=12 αL, n=10 αL NKL-L16, n=9 αM, n=8 ß2). White bars: IL-2 was added during the 24h freshly isolated day (n=7 αL, n=3 αL NKL-L16, n=5 αM, n=4 ß2). **(B and C) M**ean data and standard deviations are represented. Two tailed paired Student's t test analysis of the logarithm of raw data was used.*p<0.05, **p<0.01, ***p<0.001.

The decrease in the expression of LFA-1 observed by flow cytometry agreed with the previous observations made in experiments of confocal microscopy **(**Fig.11-D**)**. In the majority of the experiments performed, ionomycin treatment of NK cell lines was associated with a decrease in their expression of integrins. In some experiments (2/14 donors analyzed when stained for αL, 4/13 for αM, and 4/11 for ß2) integrin expression was not affected, indicating that although integrin decreased expression may have an important role in NK cell hyporesponsiveness, ionomycin treated cells must have another defect which impairs their functionality. However for all the donors stained with the NKI-L16 antibody (n=10), a decrease in the reactivity of this antibody with ionomycin treated cells was observed. When

all the experiments are considered (including those cases without integrin decreased expression, a significant decrease of αL expression (both open conformation and total) is observed after exposure to ionomycin **(**Fig. 14-B**)**. For analysis of these data, the mean fluorescence intensity (MFI) of each molecule was quantified and related to the MFI of its isotype control. The final graphs show the MFI of the ionomycin treated NK cells relative to the DMSO staining (DMSO staining considered as a 100% for each molecule). If only those cases where we observed a decrease in the integrin expression are considered, the differences were significant also for αM and ß2, the reduction of αM, αL and ß2 subunits being very similar, between 41.06% and a 51.1% **(**Fig. 14-C grey bars**)**. Although αM expression was decreased in ionomycin treated cells, its expression was very low, and the variability between different samples was higher.

Culture of the ionomycin treated cells in IL-2 led to a statistically significant recovery in the expression levels of the αM and αL molecules in those cells which had suffered a decrease in integrin expression **(**Fig. 14-C white bars**)**, although in general they didn't quite reach the same levels of expression as control cells treated with IL-2 (considered as 100% for each molecule). The increase in integrin expression driven by IL-2 is higher for ionomycin-treated cells, a mean increase of 27.34% in control cells, but a 140.26 % in ionomycin treated cells (data not shown). The expression of ß2 integrins and the NKL-L16 epitope were increased after IL-2 stimulation, but remained significantly lower than control cells.

The expression of other adhesion molecules, such as CD11c (integrin subunit αX, which also associates with the ß2 chain), CD29 (integrin subunit ß1), CD61 (integrin subunit ß3), CD44 glycoprotein, ICAM-1 glycoprotein (CD54, whose receptor are LFA-1 and Mac-1), CD44 glycoprotein and CD58 (LFA-3, a ligand for CD2), did not differ significantly between control and ionomycin treated NK cells.

These defects in integrin expression and activation could imply defects in NK cell migration and arrival to the site of inflammation, but also defects in recognition and adhesion to target cells and so NK cell activation, which could explain the lower levels of conjugates and cytotoxicity observed. The reduced integrin expression could also explain why the degranulation machinery was activated when stimulated directly by PMA/Ionomycin, but not by exposure to target cells. However, although the levels of αM, αL and ß2 integrin subunits were significantly decreased in the majority of ionomycin treated NK cells analyzed **(**Fig.14-C**)**, the levels of expression, especially those of the αL subunit, were still considerable **(**Fig.14-A**)**, making it difficult to imagine that this reduced expression of integrins was the sole basis of the observed defects in NK cell recognition.

3.3 Cytokine receptors

One possible explanation for previous data that ionomycin treated NK cells responded poorly to IL-12 and IL-18 stimulation **(**Fig.6 and Fig.7**)** could be that ionomycin treated NK cells were not sensing IL-12 and IL-18 properly, due to deficient expression of the IL-12 or IL-18 receptors. The IL-18 receptor is a heterodimeric receptor composed of an alpha (IL18R1) and a beta (IL18RAP) chain and functions to enhance NK cell IFN-γ production (Chaix et al. 2008) and cytotoxicity by both lytic granule release (Hyodo et al. 1999) and Fas-L upregulation (Tsutsui et al. 1996). Ligation of the IL-12 receptor also promotes IFN-γ production (Sareneva et al. 2000; Ferlazzo et al. 2004) and NK cell killing (DeBlaker-Hohe et al. 1995). Moreover, IL-12 signaling can also induce the expression of IL-18R subunits (Sareneva et al. 2000) demonstrating crosstalk between these cytokines. Splenocytes from mice deficient in IL-18R subunits are unable to produce IFN-γ in response to IL-12 stimulation and their NK cells are inefficient killers (Hoshino et al. 1999; Cheung et al. 2005), similarly NK cells from IL-12Rß1 deficient patients are also reported to be ineffective killers (Anfossi et al. 2006). In the context of NK cell responsiveness, deficiencies in degranulation and IFN-γ production in response to IL-12 and IL-18 priming have been described in hyporesponsive human KIR-NKG2A- NK cells (Anfossi et al. 2006), and in murine NK cells chronically stimulated through the activating receptor Ly49H (Bolanos and Tripathy 2011). These observations indicate that a defective response to these cytokines, which have important roles in NK cell function, may be a common feature of hyporesponsive NK cells.

The expression of the IL-18Rα subunit was studied by flow cytometry. In normal NK cells, the expression of IL18-Rα was very low, therefore as the differences in degranulation and IFN-γ production described previously were measured after IL-12 + IL-18 stimulation during the resting day **(**Fig.6 and Fig.7**)**, and stimulation with these cytokines is able to increase the expression of IL-18Rα (Sareneva et al. 2000), the expression of IL-18Rα after treatment with IL-12 + IL-18 was also studied. When NK cells were stimulated with IL-2 or IL-12 the levels of expression of IL-18Rα increased slightly in both control and ionomycin-treated cells **(**Fig.15-A**)**. Treatment with IL-18 alone did not increase IL-18Rα expression, but when cells were stimulated with the combination of IL-12 + IL-18, IL-18Rα expression was again increased. Indeed this increase was higher than that produced by treatment with IL-12 alone, as expected from the literature (Sareneva et al. 2000). These data indicate that both cytokines were being sensed properly and were able to synergize. Stimulation with IL-2 plus IL-12 caused the maximum increase in IL-18Rα expression **(**Fig.15-A**)**, however no significant differences were noted between control and ionomycin treated cells under any of the conditions tested. in parallel the expression levels of the IL-18RAP subunit in these experiments were also analyzed by qRT-PCR, and again no significant differences were noted between control and ionomycin treated cells **(**Fig.15-B**)**.

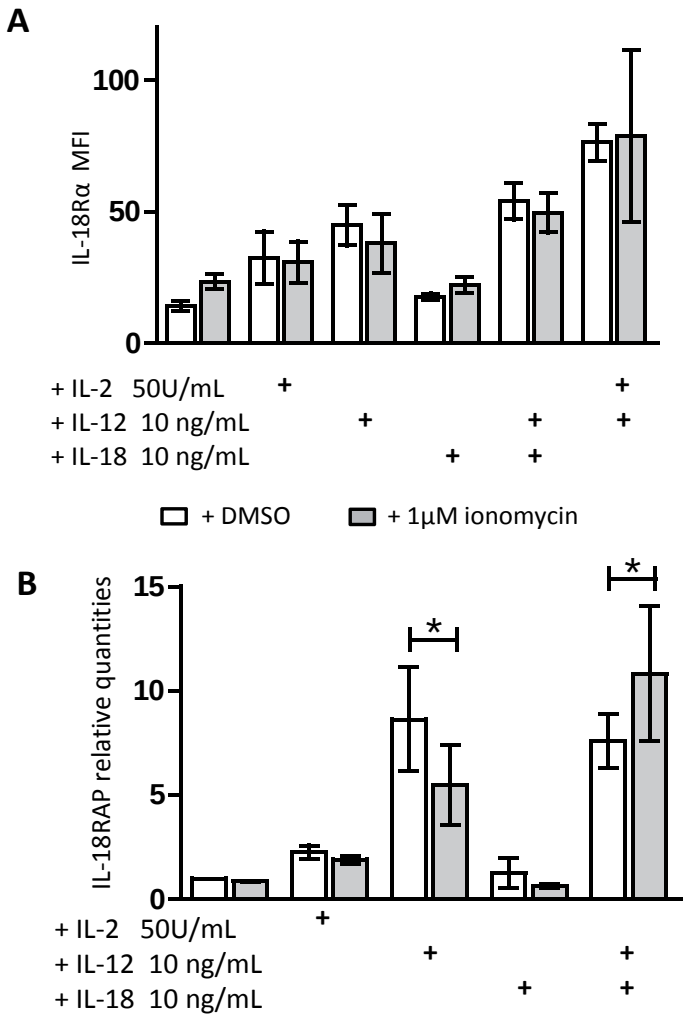


Fig. 15: Ionomycin treated NK cells expressed normal levels of IL18Rα, and IL-18 and IL-12 were able to signal efficiently. (A) Mean fluorescence intensity data from flow cytometry analysis of control and ionomycin treated NK cells after treatment with different cytokines during the rest day. (n=4) **(B)** Relative quantities or fold change of the IL-18RAP subunit as measured by quantitative PCR. Data were corrected with the 18S mRNA expression of each sample, and related to the IL-18RAP expression in control cells without cytokine stimulation using the ΔΔCt method.(n=2). **(A** and **B)** Mean data and standard deviations are represented. Paired two way ANOVA analysis and Bonferroni posttest. *p<0.05

These data suggest that the signaling pathways activated by IL-12 and IL-18 were working efficiently in ionomycin treated NK cells, and although only the expression of the IL-18R subunits were measured in these experiments, the levels of expression of IL-12R subunits must be normal too, as stimulations with IL-12 alone or in combination with IL-18, were able to increase the expression levels of IL-18Rα and IL-18RAP to the same levels as those observed in control cells.

The signaling pathways initiated by IL-12 stimulation end in STAT4 activation and increased transcription of a range of genes involved in cytokine recognition including IFN-γ and TNF-α, or EGR2 as well as the IL-12 and IL-18 receptor subunits (Good et al. 2009). IL-18 promotes AP-1 and NF-κB translocation to the nucleus synergizing with the IL-12 activating pathway (Nakahira et al. 2002). Other factors such as IκBζ also have roles associated with IFN-γ transcription (Kannan et al. 2011). Since IL-12 and IL-18 stimulation induces IL-18R subunit expression, the defect in IFN-γ production after stimulation with these cytokines is unlikely to be due to some deficiency in signaling from these receptors, but more probably reflects defects in transcription, translation or instability of the IFN-γ mRNA or protein.

When the expression of IFN-γ mRNA was analyzed, a high degree of variability between different experiments was found **(**Fig.16-A and B**)**. When the ΔΔCt values were calculated normalizing the data by the amount of IFN-γ mRNA at basal conditions in control or in ionomycin treated cells respectively, it appeared that all cytokines were able to induce *Ifng* gene transcription with similar efficiencies in both control and ionomycin treated cells, and although with 2 of the 4 cell lines analyzed, ionomycin treated cells responded less efficiently than control cells, in other cases it was the contrary (2/4), as IFN-γ mRNA was induced similarly in both cases **(**Fig.16-A**)**. If the data were normalized relative to the amount of IFN-γ mRNA in control cells without any cytokine stimulation, more variability between samples was observed, however in this analysis most of the ionomycin treated NK cells appeared to produce much lower amounts of IFN-γ mRNA than controls, although in some cases the total levels of *IFN-γ* mRNA detected were comparable **(**Fig.16-B**)**. Thus the conclusion from these analyses is that ionomycin treated NK cells were able to induce transcription of the Ifng gene efficiently, although in some cases this induction was not enough to arrive to the same final levels of mRNA found in control cells.

The expression of the IL-2Rα chain (CD25) was also analyzed, but no differences were noted in the expression of this molecule between control and ionomycin treated NK cells.

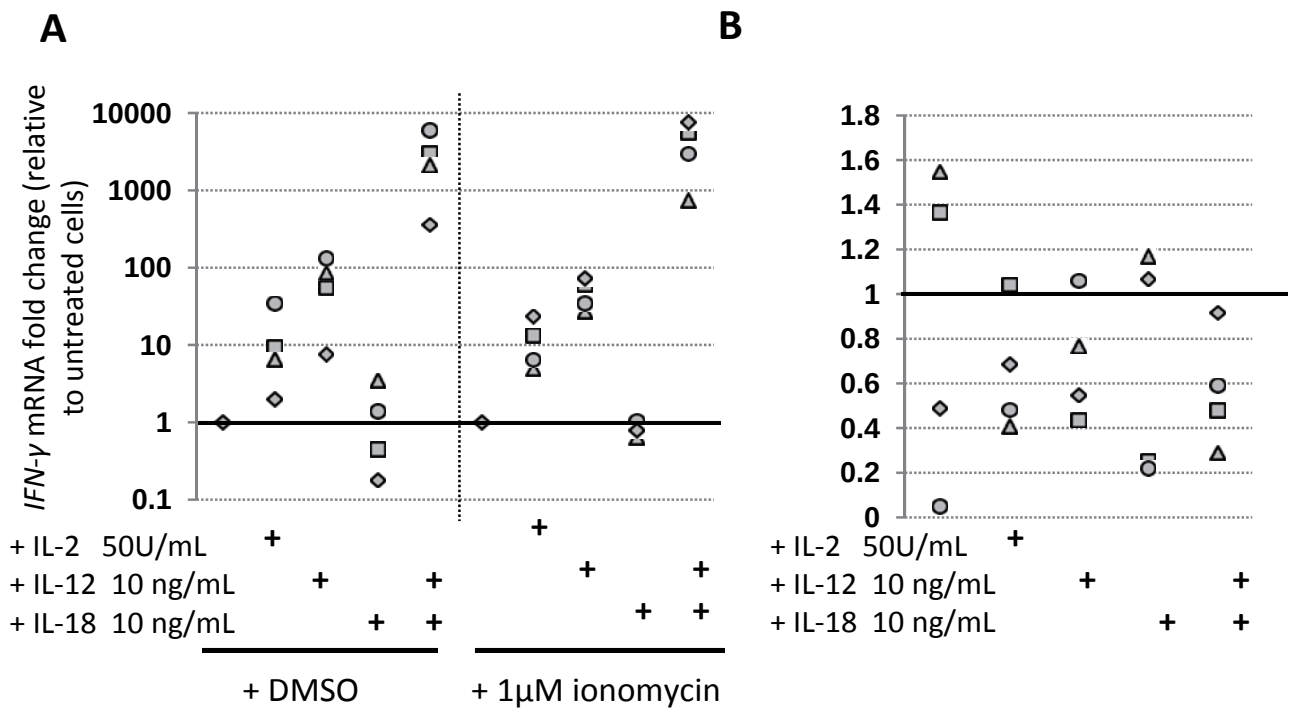


Fig. 16: Ionomycin treated NK induced IFN-γ mRNA as efficiently as control cells. (A) Relative quantities or fold change in Ifng gene mRNA measured by quantitative PCR. Data were normalized with the 18S mRNA expression of each sample, and related to Ifng gene mRNA expression on cells (DMSO or ionomycin treated) without any cytokine stimulation using the ΔΔCt method. **(B)** Relative quantities or fold change in Ifng gene mRNA measured by quantitative PCR. Data were corrected with the 18S mRNA expression of each sample, and related to the Ifng gene mRNA expression of control cells at the basal condition using the ΔΔCt method, and expressed as the relative amount in control vs. ionomycin treated cells (control =1 for each point). (n=4) **(A** and **B)** Each symbol corresponds to one of the different cell lines analyzed. (n=4). Two way Anova analysis and Bonferroni posttest. No significant differences were found.

3.4 Other molecules

A range of other receptors which have different roles in NK cell biology were analyzed, but no differences in expression between control cells and ionomycin treated NK cells were noted for CD28, CD45, CD53, CD59, CD40, CD69, CD86, HLA-DR, HLA-ABC and Fas Receptor CD95.

However, one consistent result observed in all the experiments performed was the downregulation of the expression of the CD56 molecule (NCAM) **(**Fig. 17**)**. CD56 is a glycoprotein of the Immunoglobulin superfamily widely used as a cell marker for NK and NKT cells in humans, although it is also expressed by neurons, glia and skeletal muscle cells. Although no function of CD56 has been found for NK cells, in neurons it plays important roles in cell-cell adhesion by homophilic interactions in cis or in trans, and as a receptor of soluble factors such as GDNF (glial cell line-derived neurotrophic factor) (Paratcha et al. 2003), heparin sulphate or L1-CAM. Ligand binding by NCAM can lead to the transmission of signals by activating Fyn and FAK kinases. Its expression is also related to tumor growth (Liu et al. 2011)**.** Circulating NK cells are classified as either $CD56^{bright}$ or $CD56^{dim}$ cells; the $CD56^{dim}$ population is the most abundant in blood and is related to a later step on NK cell differentiation. $CD56^{dim}$ cells are also described as more potent effector cells compared to $CD56^{bright}$ cells (Moretta 2010)**.** In the in vitro cultured NK cells maintained in IL-2 used in these experiments, only one population of very bright CD56+ cells was observed, and after ionomycin treatment, NK cells expressed lower levels of this molecule **(**Fig. 17**)**. The function of CD56 on NK cells has not yet been clearly defined, since blockade of this molecule with antibodies has no effect in cell killing (Lanier et al. 1991), although CD56 expression is thought to be related to NK-NK adhesion as transfection of Jurkat cells with this molecule causes the formation of cell-aggregates (Paratcha et al. 2003). Enrichment of a $CD56^{-}$ $CD16^{+}$ population with a decrease of the $CD56^{dim}CD16^{+}$ population has been described in patients suffering chronic HIV-1 and HCV infections. These cells secrete lower levels of cytokines, and are less cytotoxic and proliferative, and whether long term IL-2 restores their functionality is still unclear (Bjorkstrom et al. 2010), but the basis of the lower response of these cells, and whether the loss of CD56 expression has any role in this loss of function, have not been studied yet. Since CD56/NCAM1 has signaling and adhesion functions, perhaps under some circumstances of stress, this molecule could exert some role in NK cell activation and proliferation.

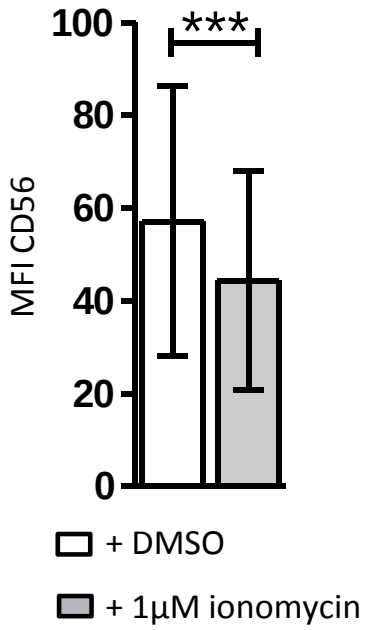


Fig. 17: Ionomycin treated cells express lower levels of CD56. CD56 mean fluorescence intensity of control and ionomycin treated NK cells. Mean data and standard deviations are represented. (n=64). Two tailed paired Student's t test analysis .**p<0.001.

3.5 Co-analysis of the expression of different receptors and NK cell degranulation ability

Treatment with ionomycin renders the majority of the NK cells in a culture unable to degranulate or produce cytokines in response to target cells, however a fraction of the NK cell population are still able to mobilize CD107a to the cell surface after exposure to target cells. This "ionomycin-resistant" population could reflect a preferential effect of the ionomycin treatment on different NK cell subpopulations. Alternatively, given that NK cells show a spectrum of responsiveness, ionomycin treatment could act to modulate the NK cell activation threshold, so that many cells would be unable to reach their activation threshold (rendered unresponsive), while other cells were still able to respond to target cell stimulation. To test whether the ionomycin treatment differentially affected different NK cell populations, degranulation experiments were performed as described before, but the staining of another receptor in the analysis was incorporated into the experiment so that the Lamp1+ and Lamp1– marker populations could be compared between control and ionomycin treated cells. For these experiments the molecules analyzed were CD11b, KIR2DL1 (CD158a), KIR2DL2/DL3 (CD158b), CD16, CD57, CD8, CD69, HLA-DR and NKG2C that are known to be expressed by only a proportion of NK cells, or whose expression varies with activation/maturation of NK cells.

No significant differences were noted on the distribution of these markers between degranulating and not degranulating NK cells in both control and ionomycin treated cells. Ionomycin treated NK cells expressed lower levels of CD11b as described before **(**Fig. 14**)**, however, they still conserved a homogeneous distribution of this molecule in $Lamp1^+$ and $Lamp1^-$ NK cells **(**Fig. 18**)**. Thus these data argue against the hypothesis that ionomycin treatment affects different subpopulations of NK cells differently.

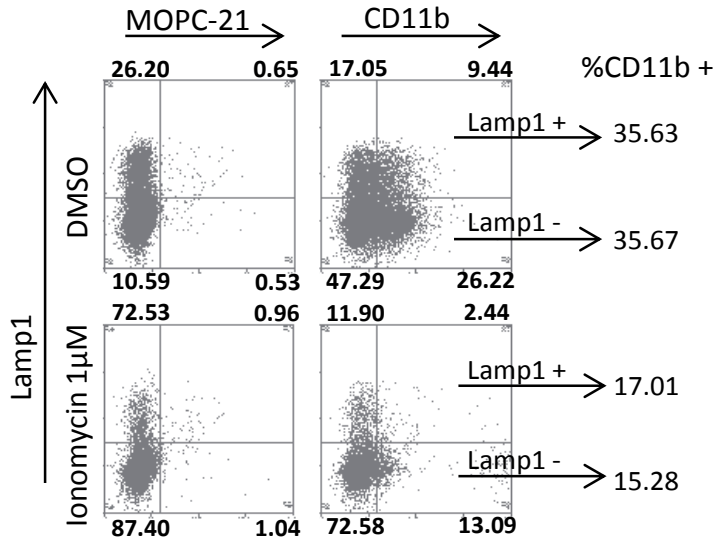


Fig. 18: Different cell markers were homogenously distributed between $Lamp1^+$ and $Lamp1^-$ NK cells. Control and ionomycin treated NK cells were cocultured for 2h with K562 target cells. Cells were stained for CD56, and in the CD56+ population coanalyzed for Lamp1 and different surface markers. Here is shown the IgG1 isotype (MOPC-21) and CD11b staining.

3.6 Redirected antibody dependent cytotoxicity

Although NK cells express a large variety of activating receptors, only CD16 is able to activate cytotoxicity by freshly isolated NK cells when stimulated alone, and receptors such as NKG2D, NKp46, DNAM-1 or 2B4 need to cooperate synergistically in order to induce an effective response (Bryceson et al. 2006).

To analyze the effect of ionomycin treatment on NK cell responsiveness in more detail, redirected antibody dependent cytotoxicity experiments using mAbs for specific NK activating receptors and P815 cells were used. P815 is a mouse lymphoblast like mastocytoma cell line, relatively resistant to NK cell mediated cytotoxicity, which expresses high affinity Fc-antibody-region receptors. When NK cells are co-cultured with the $Fc\gamma R^+$ target cell line P815 in the presence of antibodies against NK cell receptors, antibodies will attach to P815 cells through Fc receptor-antibody interactions, and to NK cells through antibody-antigen interactions; thus the antibodies act as a bridge and crosslinker to trigger NK cell activation via specific receptors. Using this approach, NK cell responses through the NKG2D, NKp30, NKp46, CD16 receptors and different combinations of them were analyzed. Two types of readout were used for this assay: 1. propidium iodide staining of labeled target cells to directly study target cell killing and 2. flow cytometry to analyze the mobilization of Lamp1 to the NK cell surface.

As cell lines from different donors made different responses after stimulation via different receptors, figure 19 shows the data from 3 different cell lines as examples of different cell responsiveness to stimuli. In these examples, we used different y axis scales to observe better the differences between control and ionomycin treated cells with the same antibody stimulation. Of the three lines represented, NK1 and NK2 cells were much more effective killers than NK3; P815 cells loaded with a CD16-specific mAb were killed efficiently by both NK1 and NK2 cells, but lysis of these cells by NK cells from donor NK3 was at much lower levels. Strikingly treatment with ionomcyin, has very little effect on CD16-triggered of cells from the donors NK1 and NK2, whereas NK3 ionomycin treated cells did not respond to this stimulus **(**Fig.19 second column**).** In contrast when antibodies against NKG2D were used to trigger lysis, ionomycin treatment markedly reduced the ability of the three cell lines to kill P815 cells as well as control cells **(**Fig.19 third column**)**. NKp30 antibodies were poor stimuli for triggering lysis by NK3 and NK1 cells. However untreated NK2 cells efficiently killed P815 cells loaded with anti-NKp30 antibodies, but ionomycin treated NK2 NK cells were unable to lyse these target cells **(**Fig.19 fourth column**)**. When NKG2D-specific mAb was used in combination with NKp30 specific antibodies, cytotoxicity of P815 cells was increased in the three cell lines compared with killing elicited by NKG2D or NKp30 antibodies alone, but killing by ionomycin treated cells was still significantly impaired **(**Fig.19 fifth column**)**. In summary, for the NK3 line, that was the least cytotoxic of the three NK lines tested, ionomycin treatment markedly reduced responsiveness to all the antibody combinations **(**Fig.19 third line**)**. For the NK1 and NK2 lines, that were much more effective killer cells, ionomycin treatment impaired killing of P815+NKG2D and P815+NKG2D+NKp30 **(**Fig.19 first line**)**, and in the case of NK2,

also of P815+NKp30 **(**Fig.19 second line**)**, but ionomycin did not significantly reduce NK lysis triggered by potent anti-CD16 stimulation.

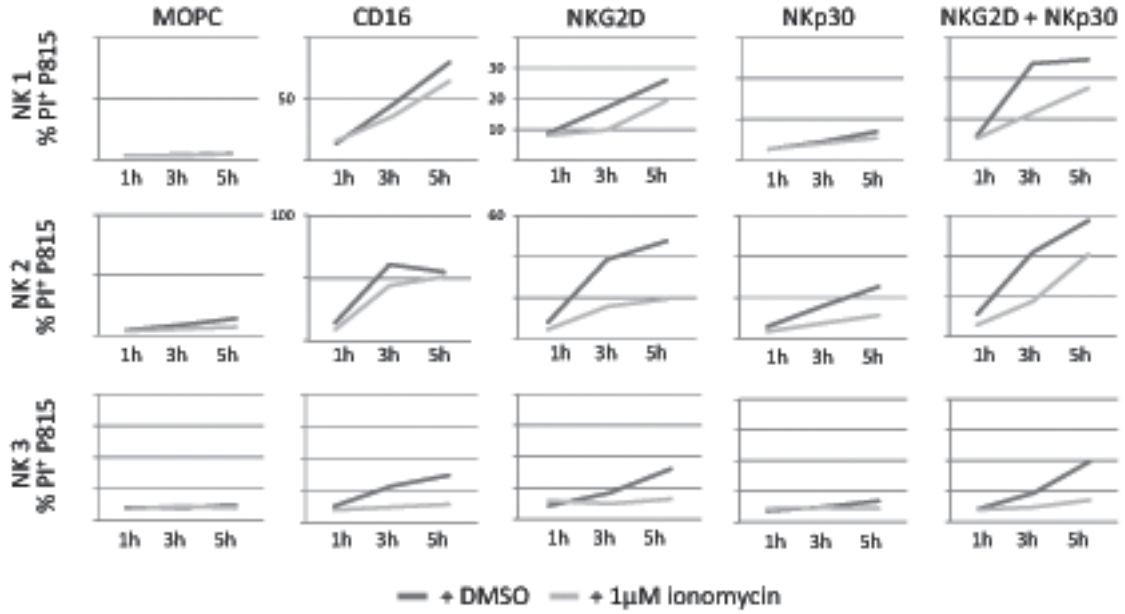


Fig. 19: Ionomycin treated NK cells were able to kill target cells efficiently when the activating stimulus was sufficiently strong. Three representative examples of flow cytometry analysis of the percentage of Propidium iodide (PI) positive P815 cells after coculture with DMSO or 1µM ionomycin treated NK cells, in combination with different antibodies, during 1h, 3h or 5 hours. The target cells were labeled with the PKH-2 dye and selected for PI analysis. Antibodies used were MOPC-21 as control, CD16, NKG2D, NKp30 and the combination of NKG2D+NKp30. Antibodies were used at 5µg/mL.

The decision as to whether or not an NK cell kills a particular target cell depends on the balance of signals from activating and inhibitory receptors interacting with their ligands on the target cell surface. When activating signals outweigh inhibitory signals, NK cells are able to respond. However, how the threshold, that the signals from activating receptors have to surpass, is established for NK cells is not well understood and very little is known about the molecular basis of how NK cells set this threshold for responsiveness. Ionomycin treatment could be affecting NK cell responsiveness by increasing the level of stimulation required to trigger efficient NK lysis of target cells. Ionomycin treated cells were unable to kill as efficiently as control cells when stimulated through intermediate stimuli as NKG2D in the three cases. on the other hand, higher stimuli as when activated through CD16 (NK1 and NK2), or lower as NKp30 (NK1 and NK3) create no differences between control and treated cells. However, each cell line has a different threshold and sense differentially different stimuli.

To further test the hypothesis that ionomycin treatment is modulating the threshold for NK cell responsiveness, NK cell degranulation after coculture with P815 loaded with specific mAbs or different combinations of antibodies was analyzed. When the NK cells were stimulated with single antibodies,

CD16 was the most potent stimulator of degranulation, and ionomycin treated cells made a response similar to that seen for control cells **(**Fig.20-A **&** B show experiments with two different NK cell lines**)**. In contrast, the response of ionomycin treated NK cells to stimulation with NKG2D alone was much weaker than that of control cells. Stimulation with NKp46 mAb triggered degranulation by control NK cells comparable to that seen with NKG2D specific mAb, but ionomycin treated cells were again somewhat less able to respond, although their response to NKp46 was not as impaired as their response to NKG2D. This experiment was repeated using NK cells from different donors and although each line showed different levels of degranulation to different receptors, the pattern of responsiveness imposed by ionomycin treatment was similar **(**Fig.20-C**)** especially for NKG2D and CD16 triggered responses. Ionomycin treatment had little effect on the degranulation response triggered by anti CD16 antibodies while the response stimulated via NKG2D was the most affected.

In this context, it was of interest to test what effect increasing the stimulation had on the ability of ionomycin treated NK cells to degranulate in response to target cells. For this purpose P815 cells were loaded with combinations of antibodies specific for receptors known to synergise for NK cell activation. However, when NK cells were confronted with P815 cells able to stimulate lysis through two different receptors known to synergize for NK cell activation (Bryceson et al. 2006), more variable results were obtained **(**Fig.20-D each symbol corresponds to a different NK cell line**)**. As expected, stimulation via NKG2D in combination with NKp30, NKp46, CD16 or 2B4, and NKp46 in combination with 2B4 triggered increased degranulation in both control and ionomycin treated cells compared to that observed after stimulation with a single antibody. However, ionomycin treated cells were still unable to respond as efficiently as control cells in most of the experiments. This experiment was also repeated using multiple NK cell lines, and although each line showed different levels of degranulation to the different receptors, the overall pattern of responsiveness imposed by ionomycin treatment was similar. Surprisingly, for two NK cell lines stimulation with 2B4 in combination with NKp46 or NKG2D was able to stimulate ionomycin treated cells as efficiently as control cells.

In aggregate, these experiments strongly supported the hypothesis that ionomycin treatment was imposing an increase in the NK cell threshold for activation so that ionomycin treated cells needed more potent stimulation to be able to respond. Thus for strong stimuli like CD16 **(**Fig.19 & 20**)**, ionomycin treated NK cells were able to degranulate as efficiently as control cells, but when the stimulation was weaker, as with NKG2D mAb alone, ionomycin treatment rendered NK cells unable to respond. Contrary to expectation, increasing stimulation by triggering NK cells with antibodies to synergistic combinations of receptors did not really overcome the effect of ionomycin treatment, however this might be explained if, apart from an altered threshold for responsiveness, ionomycin treated cells also have problems creating synergy between receptors due, for example, to changes in receptor mobility in the plasma membrane as dynamic compartmentalization of activating NK cell receptors in membrane nanodomains has been proposed to be pivotal for the control of NK cell reactivity (Guia et al. 2011).

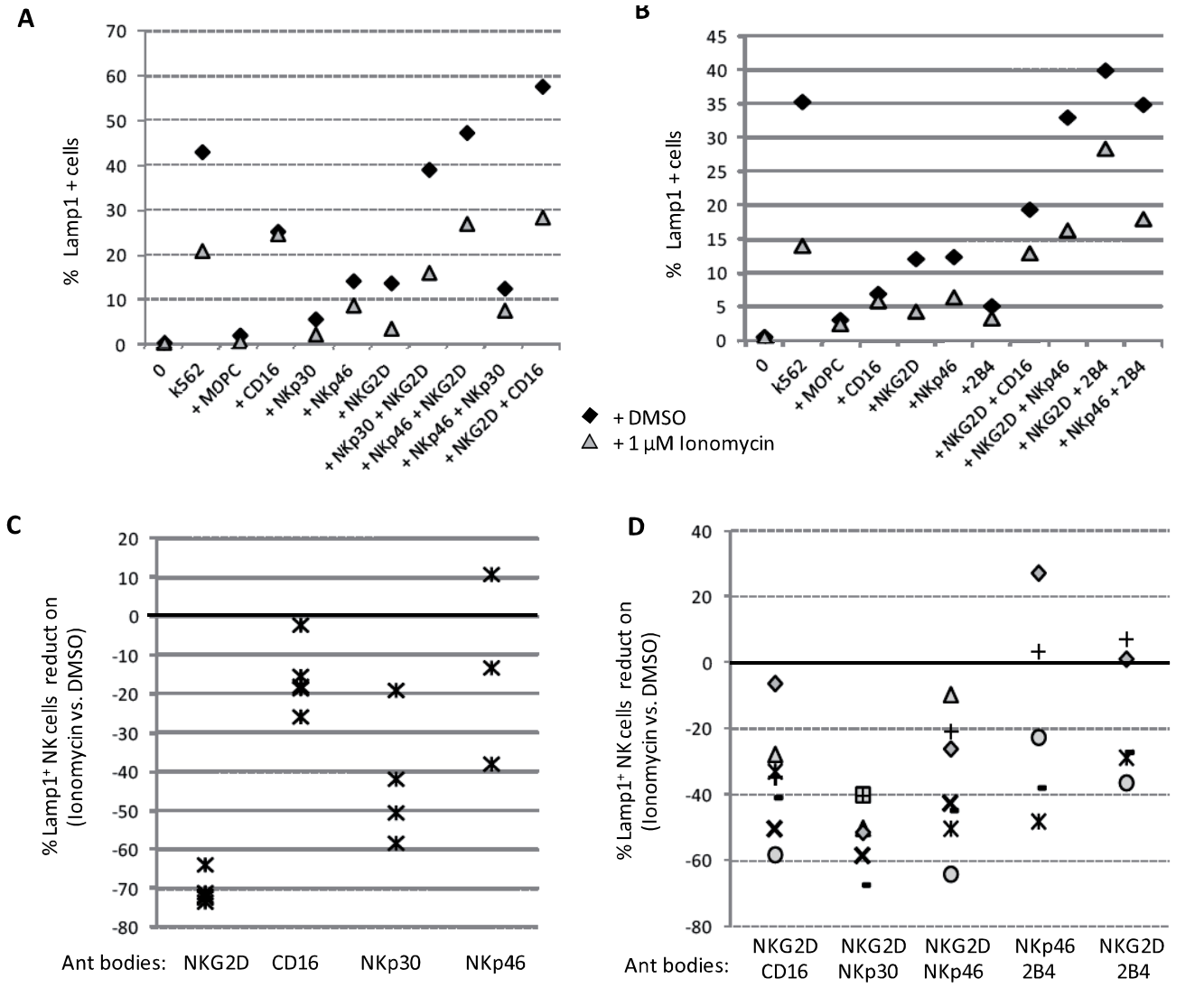


Fig. 20: Ionomycin treatment changed NK responsiveness to stimulation via different activating receptors. NK cells either untreated (DMSO) or treated with 1µM of ionomycin, were cocultured with P815 in the presence of different antibodies for 2 hours. The Fc receptors on the P815 cells were then blocked with mouse serum and the cells were stained with labeled CD56-PE and Lamp1-APC antibodies. Antibodies used for NK cell stimulation in combination with P815 cells were used at 5µg/mL each, and were, MOPC-21 (control), CD16, NKG2D, NKp30 and NKp46 alone or in different combinations**. (A** and **B)** Representative examples of the percentage of Lamp1+ NK cells after coculture with P815 and different antibodies. **(C and D)** Relative numbers of NK cells able to degranulate compared to control cells cocultured with P815 cells and different antibodies. Each symbol corresponds to one of the different cell lines analyzed.

3.7 Calcium responses

Ionomycin treated NK cells were unable to generate the same levels of degranulation when stimulated through different receptors. Previously, we have shown that ionomycin treated NK cells responses after PMA/Ionomycin stimulation were almost complete, but we wanted to confirm that the calcium responses triggered after ligation of specific receptors were comparable to control cells **(**Fig.21**)** .

Monoclonal antibodies against NKG2D and NKp46, followed by anti mouse IgG mediated crosslinking of the receptor bound mAbs, were used to stimulate the NK cells. The calcium responses of the ionomycin treated cells were slightly lower in most of the donors, although the difference was not statistically significant, probably due to the low calcium flux induced by receptor stimulation even in control cells. As a positive control for calcium signaling, cells were stimulated with 0.5µM ionomycin, but surprisingly, the response generated by ionomycin treated NK cells was also lower in most of the donors (82.18% +/- 13.56 relative to control cells maximum Ca^{2+}). One possible explanation for this result could be that the calcium stores of the ionomycin treated cells were altered, making impossible the existence of a response after receptor stimulation. To study this possibility, NK cells were stimulated with 0.5 µM ionomycin in the presence of 1mM EGTA, since under these conditions only the flux of calcium coming from the intracellular stores is visible. Using this protocol an early Ca^{2+} flux was induced, but no differences were observed between control and ionomycin treated cells. The Ca^{2+} flux occurring very early after the addition of ionomycin was similar for control and ionomycin treated NK cells when stimulated with ionomycin, while the mayor difference was observed at later times. Ionomycin is described to be a mobile ionophore, which triggers an influx of Ca^{2+} from the extracellular medium, and also allows the release of the endoplasmic reticulum calcium stores(Yoshida and Plant 1992). Ionomycin has been proposed to be able create gaps between cells (Jungmann et al. 2008), and to activate store-operated Ca^{2+} channels (SOCs) (Yoshida et al. 2010), but its mechanism is still subject to discussion. The similarity between control and ionomycin treated NK cells in the fast release of intracellular Ca^{2+}, suggests that the observed differences in the cell response to ionomcyin are due to differences in the entry of extracellular Ca^{2+} later in the response which could reflect differences in the membrane composition, or some as yet unknown mechanism by which ionomycin opens membrane channels late in the response

Previously, it has been described that the confinement of activating receptors in actin meshworks was responsible for the lower responses generated by uneducated NK cells (Guia et al. 2011). To study the possibility that actin meshworks were responsible for the lower calcium responses of ionomycin treated cells, we treated NKG2D-NKp46 stained NK cells with 1µM Latrunculin B which blocks the formation of F-actin. Treatment of both control and ionomycin treated NK cells increased the Ca^{2+} flux generated, by receptor crosslinking, about three times compared to the response seen in the absence of latrunculin (3,21 +/- 1.52 times in control cells, 2.78 +/- 0.99 in ionomycin treated cells), but the difference between control and ionomycin treated cells was still obvious. This result implies that the presence of actin meshworks limits the response of both control and ionomycin treated cells, but that other factors underly the reduced response of ionomycin treated NK cells.

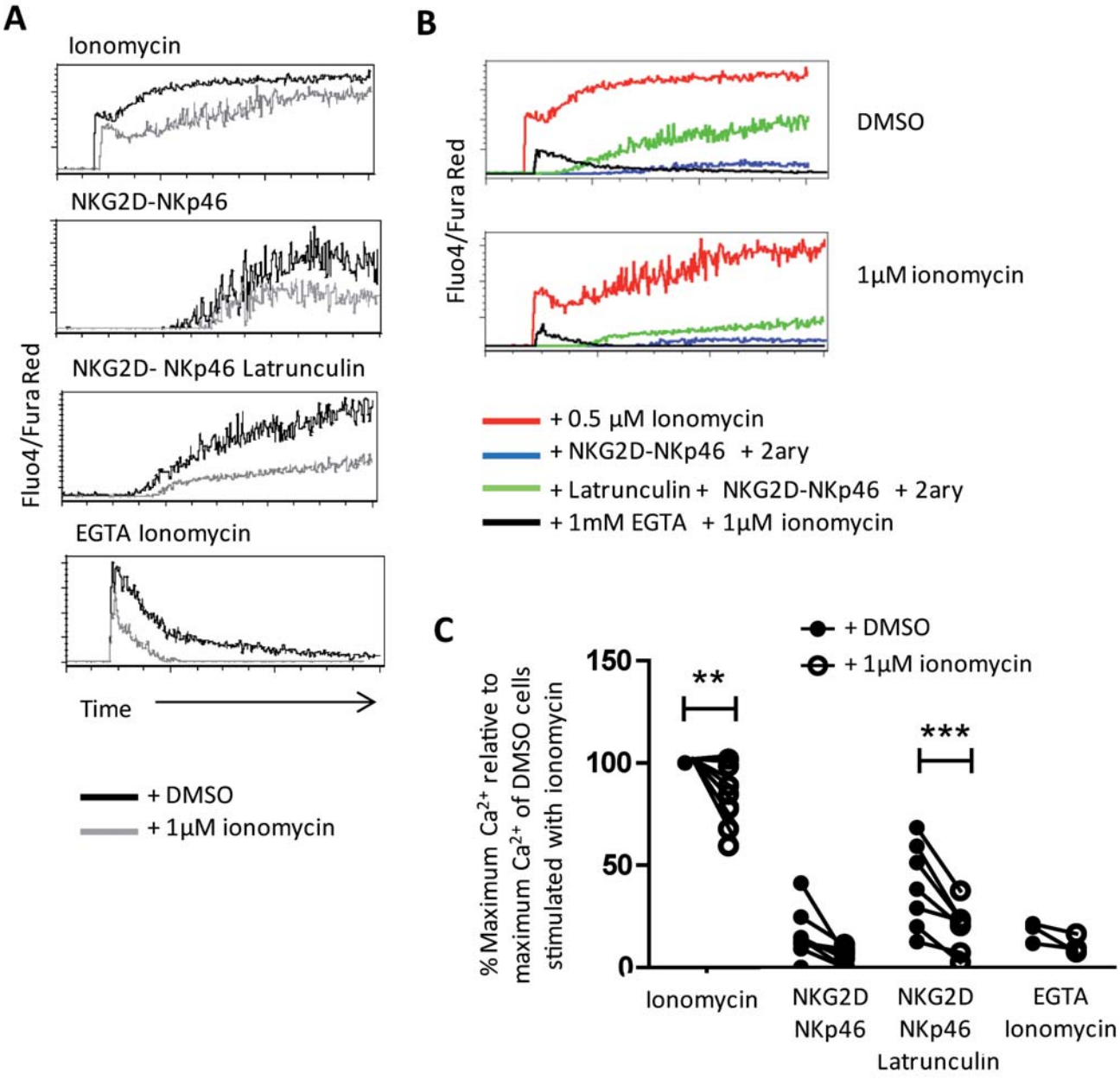


Fig. 21: Ionomycin treated NK cells showed lower Ca^{2+} flux both in response to ionomycin, and in response to NKG2D-NKp46 synergic stimulation. (A) Representative example of the flow cytometry analysisis of the Calcium flux detected by the ratio Fluo-4/Fura Red. For studying NKG2D-NKp46 triggered response NK cells were stained with 10µg/mL of the NKG2D (149810 clone) and NKp46 (9E2 clone) mAbs, on ice, and then bound mAb were crosslinked by the addition of 10µg/mL Goat anti-mouse F(ab')2. Where indicated ionomcyin (final concentration of 0.5µM) was used as a control stimulus, or 1mM EGTA was used to chelate extracellular Ca^{2+}. Black line: DMSO control cells. Grey line: 1µM ionomycin treated cells. **(B)** Overlay of the same data shown in A, Top DMSO control. Lower 1µM ionomycin treated cells. (C) Maximum Ca^{2+} levels achieved after each stimulation, relative to the maximum $Ca2^{+}$ levels induced by ionomycin in DMSO treated cells. n=8 Ionomcyin, NKG2D-NKp46. n=7 Latrunculin NKG2D-NKp46, n=3 EGTA Ionomycin. Two way Anova and Bonferroni post-test analysis was used to calculate statistical significance. **p<0.01, . ***p<0.001.

4. Ionomycin treatment of freshly isolated NK cells

In all the previous experiments, NK cells that had been cultured in IL-2 were studied as this protocol allowed the preparation of a large number of NK cells from the same donor for use in multiple experiments. However, it was of interest to confirm that the ionomycin induced hyporesponsiveness was not a feature only of activated NK cells and so some of the experiments were repeated using freshly isolated NK cells (fNK).

4.1 fNK cell receptor expression after ionomycin treatment

The expression of a number of different activating and inhibitory receptors, and molecules of the integrin family was evaluated in fNK cells following ionomycin treatment. No reproducible difference in the expression of inhibitory receptors was found; however a consistent decrease in the expression of NKG2D and CD56 was observed **(**Fig.22**)**. For this reason, for further functional experiments the MHC class I deficient human 722.221 cells was used instead of K562, as K562 killing has been described to be dependent on NKG2D ligands (Li et al. 2008), while 722.221 killing depends more on 2B4 (Sandusky et al. 2006), which was not differentially expressed between control and ionomycin treated fNK cells. The expression of αL and ß2 integrin chains was reduced in ionomycin treated fNK cells, however, this reduction was much less than that observed for activated NK cells **(**Fig.22-A**)**.

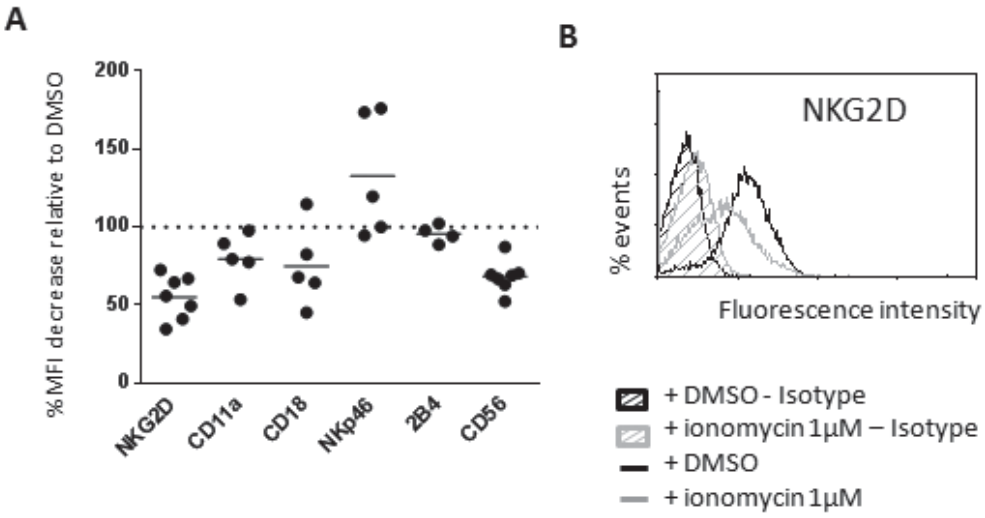


Fig. 22: Ionomycin treated fNK expressed lower levels of NKG2D.(A) Relative change in expression (MFI) of various receptorsin ionomycin treated fNK cells relative to their expression on control, DMSO treated fNKcells. **(B)** Representative histograms of the effect of ionomycin treatment on NKG2D expression. Isotype control: lined. Control cells: black line. Ionomycin treated cells: grey line.

4.2 fNKcell function after ionomycin treatment

To characterize the effects of ionomycin treatment on fNK cell function, NK cell degranulation and IFN-γ production after different cytokine stimuli were studied as described previously, using 721.221 as target cells **(**Fig. 23-A**)**. fNK showed a reduced ability to degranulate after ionomycin treatment. Stimulation with 50U/mL of IL-2 during the resting day, increased the degranulation ability of NK cells from all the donors, although not always to the levels of degranulation of control cells **(**Fig. 23-A**)**. This incomplete restoration was particularly obvious when IFN-γ production was analyzed, where none of the ionomycin-treated NK cells analyzed were able to produce as much IFN-γ as control cells **(**Fig. 23-C**)**. Interestingly, donors where IL-2 treatment restored the same levels of degranulation as control cells showed clearly that weren´t rescued for IFN-γ production by these cells was not rescued.

Similarly to activated NK cells, fNK cells treated with ionomycin also didn't efficiently produce IFN-γ when they were stimulated with IL-12 and IL-18 (10ng/mL of each) during the resting day **(**Fig. 23-C**)**. These differences in the recovery of their function between activated and freshly isolated NK cells, could indicate either that prior activation has altered the NK cells, so that IL-2 treatment can rescue the ionomycin induced defect in degranulation and IFN-γ production or that fNK cells are much more susceptible to the effect(s) of ionomycin. Another possibility is that IL-2 is not rescuing all the possible defects induced by ionomycin treatment that contribute to the hyporesponsive phenotype, but rather acts by bypassing some of the defective stages in activated but is unable to do so in fNK cells.

As IL-2 stimulation did not produce a complete recovery of NK cell function, it was of interest to test if ADCC was functional, or not, in fNK treated with ionomycin. Redirected antibody mediated cytotoxicity was evaluated using P815 cells and mAbs to stimulate either the CD16 receptor or a combination of the NKp46 and 2B4 receptors. In contrast to activated NK cells, fNK cells weren't as able to degranulate after CD16 stimulation as control cells, and although the percentage of degranulating cells were high, they never reached the levels of degranulation observed for control cells **(**Fig.23-B**)**.

Calcium responses were also evaluated on fNK cells after crosslinking of the activating receptors NKp46 and 2B4. The response to ligation of this pair of receptors was much higher than that observed previously with NKG2D and NKp46 on activated NK cells. The calcium response was, in all the donors tested, slightly lower in ionomycin treated NK cells. However it was still a very potent response. Moreover, in contrast to activated NK cells, treatment with 1mM Latrunculin B induced only a very low increase in the response in comparison with the Ca^{2+} flux generated in the absence of latrunculin (1.24 +/- 0.26 times in control cells, 1.46 +/- 0.36 in ionomycin treated cells). The response generated by 0.5 µM ionomycin was also different between control and ionomycin treated cells (93.11 +/- 8.45 56 relative to control cells maximum Ca^{2+}), being lower in hyporesponsive ionomycin treated NK cells. However, study of the mobilization of intracellular stores of calcium after stimulation with 0.5 µM

ionomycin in the presence of 1mM EGTA, showed no difference between both groups and these data are similar to those obtained on study of activated NK cells **(**Fig.24**)**.

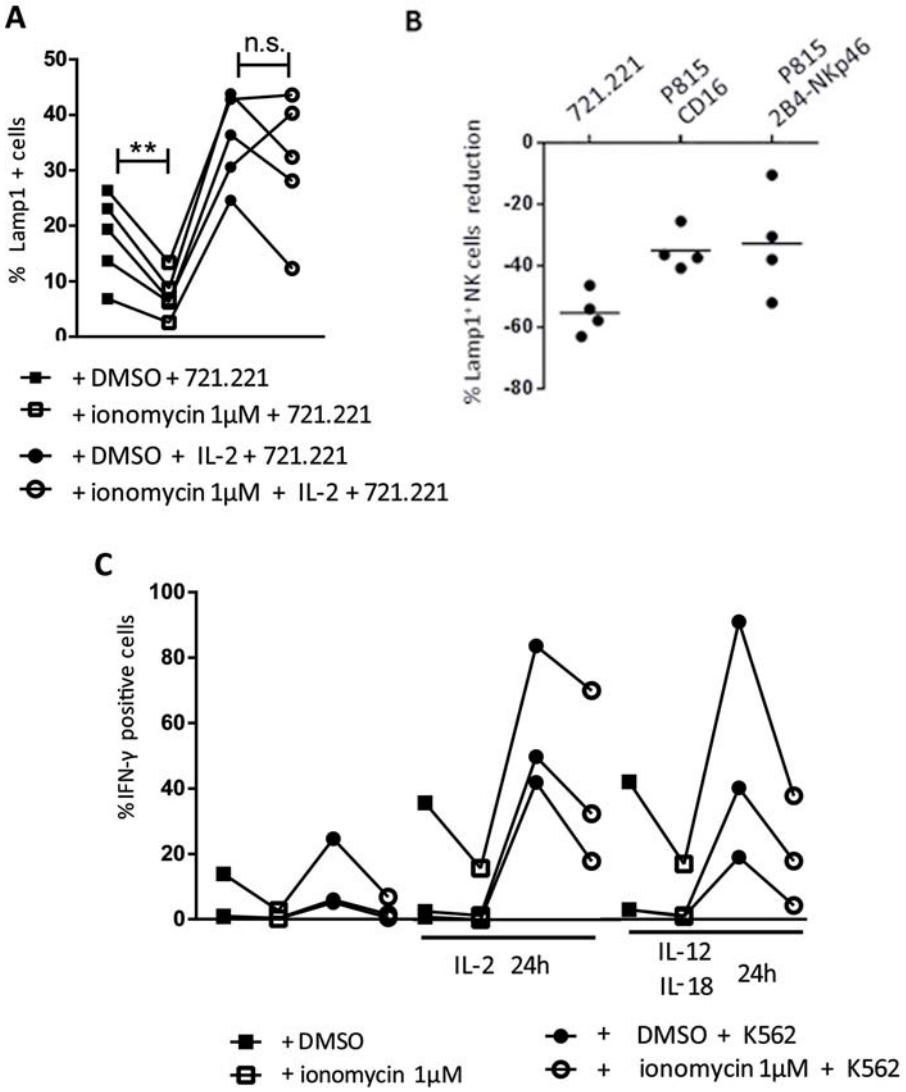


Fig. 23: Ionomycin treated fNKcells displayed a lower ability to degranulate and produce IFN-γ in response to 721.221 target cells, independently of IL-2 or IL-12 + IL-18 stimulation. (A) Flow cytometry analysis of the percentage of NK cells, untreated or stimulated with 50U/mL IL-2 during the rest day, that expressed Lamp1 on the cell surface after coculture with K562 for 2 hours. Data from five different experiments are represented. Two tailed paired Student's t test analysis was used. **p<0.01. **(B)** Reduction in the percentage of NK cells degranulating in comparison to DMSO control cells when cocultured with 721.221 target cells, P815 with 10 µg/mL of the CD16 antibody (KD1 clone) or P815 with 10 µg/mL of the 2B4 (C1.7 clone) and NKp46 (9E2 clone) antibodies for 2 hours. **(C)** IFN-γ production by NK cells was analyzed by flow cytometry. Cells were unstimulated, stimulated with IL-2 overnight or stimulated with IL-12/IL-18 overnight, and then cultured for 6 hours in the presence of 2,5 µM Monensin with/without 721.221 target cells. Three different experiments are represented.

Study of the calcium responses in ionomycin-treated fNK cells after 2B4-NKp46 stimulation, showed that although slightly lower, the calcium induction in these cells was still very efficient **(**Fig.24**)**. This observation was all the more striking since ionomycin treatment produced a marked reduction in the degranulation of NK cells with P815 cells in the presence of 2B4-NKp46 antibodies **(**Fig.24**)**. These

observations demonstrated that although there may exist some defect at the level of signaling proximal to the receptor, at least the calcium response is largely intact in these cells. Again in contrast to the observations made with activated NK cells, treatment with latrunculin didn't obviously enhance the response generated, but this may be because the response generated by the 2B4-NKp46 crosslinking is already very potent, perhaps close to the maximum limit of the Ca^{2+} flux that can be generated by the cell.

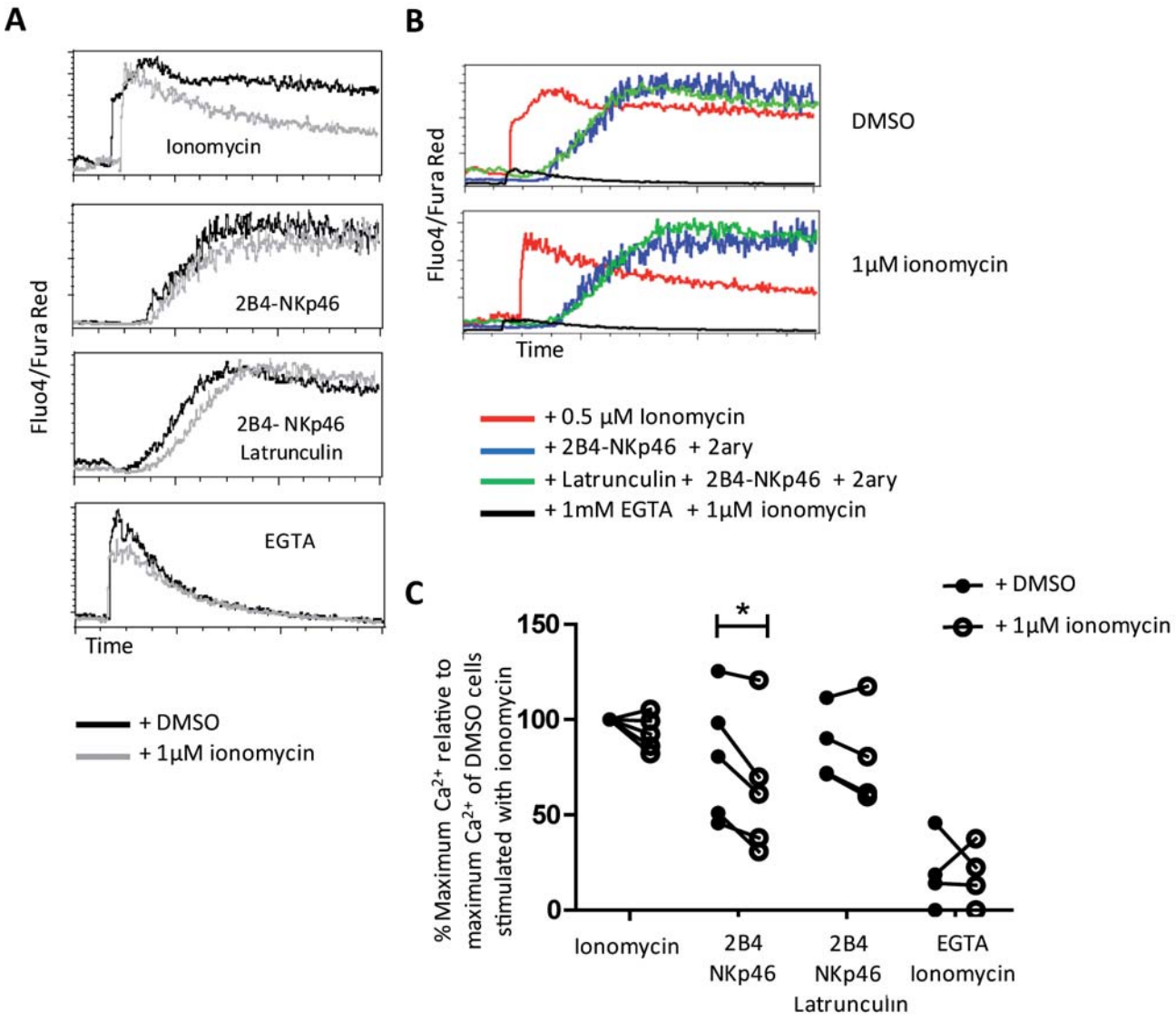


Fig. 24: Ionomycin treated fNK cells showed lower responses generated by ionomycin, and slightly lower Ca^{2+} flux in response to 2B4-NKp46 stimulation. (A) Representative example of the flow cytometryanalysisis of the Calcium flux detected by the ratio Fluo-4/Fura Red. To study the 2B4-NKp46 triggered response NK cells were stained with 10µg/mL of mAbs specific for2B4 (C1.7 clone) and NKp46 (9E2 clone) on ice, and then crosslinked by the addition of 10 µg/mL Goat anti-mouse F(ab')2 at 37oC. Where indicated, ionomcyin (final concentration of 0.5µM) was used as a positive control, or 1mM EGTA was used to chelate extracellular Ca^{2+}. Black line: DMSO control cells. Grey line: 1µM ionomycin treated cells.**(B)** Overlay of the same data shown in A, Top DMSO control. Lower 1µM ionomycin treated cells.**(C)** Maximum Ca^{2+} levels achieved by each stimulation, relative to the maximum Ca2+ levels induced by ionomycin in DMSO treated cells. n=5 Ionomcyin, 2B4-NKp46. n=4 Latrunculin 2B4-NKp46 and EGTA Ionomycin. Two way Anova and Bonferroni post-test analysis was used to calculate statistical significance. *p<0.05.

5. Transcriptome analysis of ionomycin treated NK cells

The processes of anergy (Macian et al. 2002), exhaustion (Wherry et al. 2007) or tolerance (Schietinger et al. 2012) in T cells are reflected in changes in the expression profiles of multiple genes and transcriptomics studies have been important in providing insight into the molecular mechanisms underlying these different functional states. As ionomycin treated NK cells exhibit deficient functional properties, it seemed reasonable to hypothesize that these changes in function were accompanied by changes in the pattern of gene expression. Therefore cDNA microarray experiments were performed to study in detail the changes in gene expression associated with NK hyporesponsiveness. mRNA was isolated from NK cells from 4 different donors, which had been treated with either 1µM ionomycin or the vehicle control (DMSO), and analysis of gene expression in these samples was performed by microarray using two color Agilent human gene expression 4x44 v2 chip, which allows analysis of the expression of 27,958 human genes. These data were collected and preprocessed by the CNB-Genomics Unit. Differentially expressed genes were visualized and selected from the FIESTA viewer according to the following criteria: a False Discovery Rate (FDR) < 0.05 by Rank Products analysis, and a Fold Change <= -1.6 (repressed genes) or >= +1.6 (induced genes).

From this analysis 75 genes were found to have higher expression in ionomycin treated cells (in 4/4 donors), while 72 showed lower expression compared to control NK cells.

To validate the microarray data the differential expression of a selection of the downmodulated genes was analyzed by qRT-PCR (*cd300a, cd56, ctsw, dap10, egr1, gnly, gzmh, il18r1, itgam, itgb2, matk, myom, ncr3, sell*) using mRNA from control and ionomycin treated NK cells isolated from other 6 different donors. **(**Fig. 25**)**.

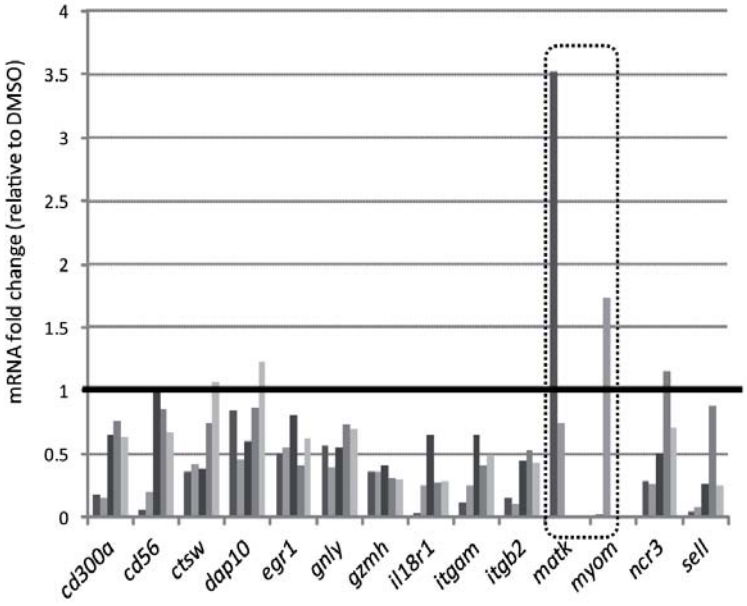


Fig. 25: Gene expression analysis by qPCR of 14 selected genes that appeared as downmodulated in the microarray experiment, in ionomycin treated cells compared with DMSO condition. (n=6). ΔΔCt method was used for quantification. All data were corrected by 18S gene expression. Highlighted data represent genes that were discarded as appeared as the downregulated observation in the microarray experiment was not confirmed in qPCR.

This qPCR analysis confirmed that most, although not all, of the genes classified as downmodulated in the microarray analysis, were downregulated in the mRNA isolated from ionomycin-treated NK cells from different donors, so validating the data obtained from the microarray experiments.

As ionomycin treated NK cells recovered functionality after being restimulated with IL-2 during the rest day, it was of interest to analyze how the expression of the genes studied in Fig. 26, was affected by IL-2 treatment. For these experiments, mRNA from 4 new donors was isolated from control and 1µM ionomycin treated NK cells, that had been stimulated or not with IL-2 during the 24 hour recovery period. IL-2 treatment rescued expression of *egr1*, *itgam* and *itgb2* genes in 4 samples **(**Fig. 26-A**)**, *ctsw* in 3 donors, and *ncam*, *dap10*, *gnly* and *ncr3* in 2 donors (not shown).

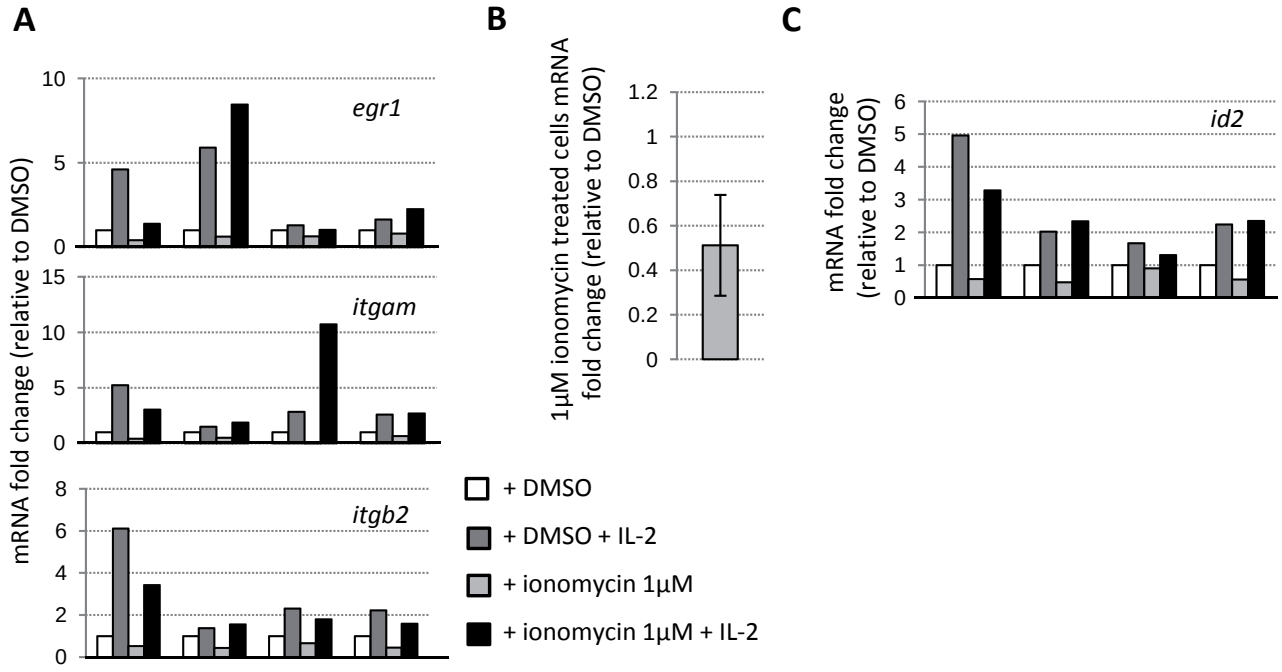


Fig. 26: egr1, itgam, itgb2 and id2 genes, recovered their mRNA expression on ionomycin treated NK cells following IL-2 stimulation. (A) mRNA from DMSO and 1µM ionomycin treated NK cells, stimulated or not with IL-2 was analyzed by qPCR for egr1, itgam,itgb2 and (C) id2 genes. **(B)** mRNA fold change of the id2 gene in ionomycin treated cells (n=6). (A, B and C) ΔΔCt method was used for quantification taking DMSO without IL-2 stimulation as control (1=DMSO). All data were normalised using 18S gene expression.

The analysis of the *id2* gene, which is a transcription factor with important functions in differentiation and development of the NK cell lineage (Ikawa et al. 2001) was also included in our analysis since although the downregulation of *id2* was not statistically significant, it was downregulated in 4/4 samples. Moreover, the expression of ID2 has been recently shown as being diminished in the *mysm1*$^{-/-}$ mice which showed normal NK cell development but altered maturation (Nandakumar et al. 2013). When *id2* gene expression was analyzed in mRNA from the 6 new ionomycin treated samples it was downmodulated in all of them **(**Fig. 26-B**)**, and its expression recovered after IL-2 stimulation in the 4 samples analyzed **(**Fig. 26-C**)**.

5.1 Gene classification by its cell function.

Gene Ontology analysis using the Genecodis http://genecodis.cnb.csic.es/ or g:profiler http://biit.cs.ut.ee/gprofiler/ suggested that several pathways or functions were enriched in the lists of genes upregulated and downregulated after ionomycin treatment. Examples of these significantly enriched pathways are presented on table 1.

Ionomycin downregulated genes	
ENRICHED PATHWAYS	
defense responses (GO:0006952)	*gimap5, mmp25, il18r1, ncr3, nt5e, ccr3, il3ra, adrb2, snca, ccr5, ccr1, ncf2, rnase6, gnly, itgb2, tnf, ccl4l1, ccl4, ccl3, ccr2*
inflammatory response (GO:0006954)	*mmp25, ncr3, nt5e, ccr3, il3ra, adrb2, ccr5, ccr1, itgb2, tnf ,ccl4l1, ccl4, ccl3, ccr2*
regulation of immune system process (GO:0002682)	*egr1, gimap5, ncr3, snca, tnf*
innate immune response (GO:0045087)	*ncam1, ltb, adrb2, tnf*
chemotaxis (GO:0006935)	*ccr2, ccl3, ccl4, ccl4l1, itgb2, itgam, ccr1, ccr5, ccr3, cmklr1*
locomotory behavior (GO:0007626)	*cmklr1, itgam, ccr3, snca, ccr5, ccr1, itgb2, ccl4l1, ccl4, ccl3, ccr2*
cellular calcium ion homeostasis (GO:0006874)	*ccr2, ccl3, cd52, ccr1, ccr5, ccr3*
cell-cell signaling (GO:0007267)	*pcsk5, tspan32, spry2, egr2, ltb, egr1, snca, ccr5, ccr1, itgb2, tnf, ccl4, ccl3*
cell adhesion (GO:0007155)	*itga6, cd300a, sell, ncam1, itgam, ccr3, ccr1, itgb2, tnf, ccl4*
integrin-mediated signaling pathway (GO:0007229)	*ncam1, ltb, adrb2, tnf*
cell activation (GO:0001775)	*ltb, itgam, egr1, gimap5, snca, tnf*
leukocyte activation (GO:0045321)	*itgam, egr1, gimap5, snca*
immunesystem development (GO:0002520)	*timp1, irf8, ltb, egr1, gimap5, tnf*
regulation of cell proliferation (GO:0042127)	*matk, ifitm1, timp1, ltb, adrb2, tnf*
cytokine production (GO:0001816)	*pcsk5, ltb, gimap5, tnf*
positive regulation of signal transduction (GO:0009967)	*ncam1, ltb, adrb2, tnf*
positive regulation of programmed cell death (GO:0043067)	*ncam1, ltb, adrb2, tnf*
ENRICHED CELLULAR LOCALIZATIONS	
cell surface (GO:0009986)	*ITGA6, ccr5, ncam1, itgam, sell, tnf, mmp25*
membrane rafts (GO:0045121)	*stom, adrb2, tnf*
extracellular space (GO:0005615)	*ltb, gnly, tnf, ccl4l1, ccl4, ccl3, ccl3l3, pcsk5*
ENRICHED FUNCTIONS	
cytokine activity (GO:0005125)	*ltb, tnf,* ENST00000400702*, ccl4l1, ccl4, ccl3, ccl3l3*
cytokine binding (GO:0019955)	*il18r1, il3ra, ncam1, cxcr7, cmklr1, ccr3, ccr5, cxcr6, ccr1, ccr2*
peptidase activity (GO:0008233)	*mmp25, gzmh, ctsw, gzmk, gzma, pcsk5*
cell death (GO:0008219)	*ltb, gzmh, gzma, itgb2, tnf*
natural killer cell mediated cytotoxicity (hsa04650)	*ncr3, hcst, itgb2, tnf*
cytokine-cytokine receptor interaction (hsa04060)	*il18r1, il3ra, ltb, ccr5, cxcr6, ccr1, tnf, ccl4l1, ccl4, ccl3, ccr2, ccl3l3*
leukocyte transendothelial migration (hsa04670)	*nf2, itgam, itgb2*

Ionomycin upregulated genes	
ENRICHED PATHWAYS	
response to organic substance (GO:0010033)	*creb3l3, cyp1b1, lef1, ptprf, igfbp2, gng4, dusp4, cd27, cav1, hspb1, hmox1*
regulation of signal transduction (GO:0009966)	*lef1, ptprf, igfbp2, p2rx5, gng4, ramp1, dusp2, cd27, cav1, hmox1*
positiveregulation of signal transduction (GO:0009967)	*p2rx5, cd27, cav1, hmox1*
positive regulation of programmed cell death (GO_0043068)	*ptprf, prkce, mal, tnfrsf9, cd27, hmox1*
dephosphorylation (GO:0016311)	*ptprf, prkce, mal, tnfrsf9, cd27, hmox1*
cellular ion homeostasis (GO:0006873)	*pmch, p2rx5, mal, cav1*
ENRICHED CELLULAR LOCALIZATIONS	
insoluble fraction (GO:0005626)	*hspb1, lmna, prkce, ptprf, ppap2a, rdh10, mal, hmox1, cyp1b1, cav1, pon3, cd27*
extracellular space (GO:0005615)	*ramp1, cd109, pmch, igfbp2, vcam1, hmox1, pon3*
vesicular fraction (GO:0042598)	*ptprf, rdh10, hmox1, cyp1b1, pon3*
ENRICHED FUNCTIONS	
phosphatases (GO:0016791)	*dusp4, ptprf, ppap2a, dusp2, pon3*
oxidoreductases (GO:0016491)	a_23_p431853, a_23_p317056, *igfbp2, pycr1, rdh10, moxd1, cyp1b1, hmox1*

Table 1: Ionomycin treatment repressed and induced different NK cell pathways. Gene ontology analysis of differentially expressed genes on ionomycin treated NK cells highlighted different pathways, cellular localization and functions. Examples of enriched features are shown in table 1.

These analyses help to understand better the nature and the consequences of the altered pattern of gene expression of ionomycin treated NK cells. The downregulated list of genes is enriched in genes related to chemotaxis, adhesion and cytotoxicity, thus it is easy to imagine that deficient expression of these genes will profoundly affect NK cell response and behavior. The upregulated list of genes is enriched in regulatory genes, phosphatases and oxidoreductases that could modulate signal transduction.

As many genes didn't appear in this analysis, differentially expressed genes were also classified manually as shown in table 2 according to their known functions in NK cells and genes related to actin reorganization, adhesion, calcium signaling, cell cycle, cell movement, chemokine and cytokine signaling, DNA-RNA synthesis, cytotoxicity, cell matrix, membrane molecules, metabolism, cell receptors, regulatory, secretion or signaling were found. Other genes (43) had no associated function yet, or cannot be included in the previous groups. Many of the genes differentially expressed have no well defined role on NK cells, and their function is extrapolated from other cell types, thus these functional associations are speculative **(**Table 2**)**.

Role	Fold change	Gene	Role	Fold change	Gene	Role	Fold change	Gene
Actin reorganization	2.94	HSPB1	dnasynthesis	-4.69	ZNF683	metabolism	3.22	CYP1B1
Actin reorganization	11.08	PLS3	dnasynthesis	-4.64	EGR1	metabolism	3.24	FABP5
adhesion	-4.03	SELL	dnasynthesis	-4.29	IRF8	metabolism	3.85	HMOX1
adhesion	-3.2	ITGB2	dnasynthesis	-3.31	XBP1	metabolism	4.2	NUAK1
adhesion	-2.84	ITGAM	dnasynthesis	-2.94	EGR2	metabolism	5.61	PON3
adhesion	-2.67	NCAM1	dnasynthesis	-2.4	IFITM1	receptor	-4.81	CD300C
adhesion	-2.27	ITGA6	dnasynthesis	2.4	LEF1	receptor	-4.24	CD300A
adhesion	-2.21	PCSK5	dnasynthesis	2.52	HOXB6	receptor	-3.2	ITGB2
adhesion	2.44	PTPRF	dnasynthesis	2.58	DMRT1	receptor	-2.84	ITGAM
adhesion	2.48	CERCAM	dnasynthesis	2.59	ZBED2	receptor	-2.74	HCST
adhesion	3.34	VCAM1	dnasynthesis	2.61	LMNA	receptor	-2.67	ADRB2
adhesion	3.5	ITGB5	dnasynthesis	2.75	TOP1MT	receptor	-2.27	ITGA6
calcium	-2.2	CPNE7	dnasynthesis	3.05	CBFA2T3	receptor	-2.27	NCR3
calcium	3.24	FLVCR2	dnasynthesis	3.39	CREB3L3	receptor	-2.24	SLAMF8
cellcycle	-2.4	IFITM1	dnasynthesis	3.41	HES6	receptor	3.45	CD160
cellcycle	2.41	NBL1	dnasynthesis	5.02	EPAS1	receptor	7.24	CD27
cellcycle	2.53	BMF	cytotoxicity	-3.25	GZMK	regulatory	-2.77	RGS18
cellmovement	-2.74	MYOM2	cytotoxicity	-3.1	TNF	regulatory	-2.63	SPRY2
cellmovement	-2.3	NT5E	cytotoxicity	-2.98	GZMA	regulatory	2.39	RAMP1
cellmovement	2.57	COL6A1	cytotoxicity	-2.97	GNLY	regulatory	2.69	PMEPA1
cellmovement	3.23	B3GNT7	cytotoxicity	-2.67	GZMH	regulatory	2.71	UCHL1
cellmovement	3.47	HPSE	cytotoxicity	-2.98	CTSW	regulatory	3.11	RNF130
cellmovement	16.69	CTHRC1	cytotoxicity	-2.63	LTB	regulatory	3.69	REEP2
chemokine and cytokine	-4.6	CCL3L3	matrix	-2.34	TIMP1	secretion	3.47	HPSE
chemokine and cytokine	-4.46	CCR2	matrix	-2.2	MMP25	signaling	-3.82	MATK
chemokine and cytokine	-3.62	CCL3	matrix	2.98	PLAU	signaling	-3.36	PTGDR
chemokine and cytokine	-3.49	CCL4	membrane	5.32	CD9	signaling	-3.3	STYK1
chemokine and cytokine	-3.46	CCL4L1	membrane	-2.46	TSPAN32	signaling	-2.74	HCST
chemokine and cytokine	-2.77	CCR5	metabolism	-3.95	HS3ST3B1	signaling	2.25	PRKCE
chemokine and cytokine	-2.68	CCR1	metabolism	-2.81	CD52	signaling	2.34	PRKCDBP
chemokine and cytokine	-2.59	CXCR6	metabolism	-2.8	NCF2	signaling	2.41	DUSP4
chemokine and cytokine	-2.53	IL3RA	metabolism	-2.74	LTC4S	signaling	2.44	PTPRF
chemokine and cytokine	-2.47	CCR3	metabolism	-2.35	GPD1L	signaling	2.49	PPAP2A
chemokine and cytokine	-2.46	CMKLR1	metabolism	-2.17	GIMAP5	signaling	2.59	CD109
chemokine and cytokine	-2.4	CXCR7	metabolism	2.49	SLC7A5	signaling	2.72	DUSP2
chemokine and cytokine	-2.23	IL18R1	metabolism	2.64	PYCR1	signaling	2.92	MAL
						signaling	4.06	CAV1
	-4.61	ENST00000443661		-2.44	UNQ6228		2.62	PMCH
Otherorunknownfunctions	-4.49	ENST00000390595		-2.38	C10orf18		2.8	A_23_P431853
	-4.48	LOC100133862		-2.35	PLAC8		2.87	LAYN
	-4.45	ENST00000390622		-2.27	APOBEC3H		2.89	RDH10
	-4.25	ENST00000390605		-2.23	STOM		2.9	MOXD1
	-3.71	A_33_P3397473		1.56	A_24_P307384		3.5	P2RX5
	-3.18	LOC100290415		2.25	LRRC28		3.57	LRRC16B
	-3	CCDC65		2.27	TUBB2A		4.01	IGJ
	-2.97	LOC100292999		2.3	LOC100008589		4.15	NMB
	-2.81	RNASE6		2.5	IGFBP2		4.21	GNG4
	-2.74	SNCA		2.56	A_33_P3253832		4.27	TNFRSF9
	-2.72	ENST00000400702		2.56	MAGEF1		4.45	C7orf57
					TMEM11			
	-2.71	PACSIN1		2.59	7		5.17	A_23_P317056
	-2.65	RGL4		2.6	A_24_P272515		9.1	KIAA1324
	-2.47	C20orf197		2.6	CR591103			

Some interesting genes found in this analysis were:

- *Adhesion molecules*: **ITGB2** (-3.20) and **ITGAM** (-2.84) downregulation correlates with the observed decreased expression observed at protein levels **(**Fig. 14**)**. The expression of **SELL** (Selectin-L) (-4.03) was also analyzed, but no difference in expression was found at protein level. Differences in mRNA expression of other integrins such as **ITGA6**, **ITGB5** or **VCAM1** were also noted, but were not analyzed further. These changes in the repertoire of adhesion molecules expressed by ionomycin treated cells could affect their ability to recognize target cells and interact with them, and also their migration pattern. The CD56 gene (**NCAM**, -2.67) appeared also as downmodulated, which correlated with its lower expression at protein levels **(**Fig. 17**).** The expression of **CD9** (5.32) was also analyzed by flow cytometry but no consistent upregulation of this molecule was observed on ionomycin treated cells at protein level.

- *Cell cycle*: The IFN induced transmembrane protein **IFITM1** (-2.40) mediates inhibition of cell cycle and ERK signaling (Yang et al. 2007).

- *Chemokine and cytokine signaling*: Many chemokine receptors and ligands appeared as downmodulated in these data, indeed this was the category of genes most enriched in the downmodulated group. Of special interest was **CCR5** (-2.77) since downmodulation of this molecule has been related to $CD8^+$ T cell tolerance (Schietinger et al. 2012), and its sequestration by regulatory T cells has profound effects on antigen recognition (Pace et al. 2012). A number of different CCR5 ligands including **CCL3** (-3.62) and **CCL4** (-3.49) were also downmodulated as was CCL5 (RANTES), another CCR5 ligand (-2.05), but here the FDR was 0.138. Lower expression of all these molecules may again imply defects in the migration pattern of these cells, but also functional defects. The IL-18 receptor α-chain gene (**IL18R1** -2.23) also appeared as downregulated but expression of this receptor as protein was normal in ionomycin treated NK cells **(**Fig. 17-A**)**.

- *DNA-RNA synthesis*: The expression of a number of different transcription factors was altered in ionomycin treated cells including **LEF1** (2.40), **EGR1** (-4.64), **EGR2** (-2.94), and this could produce differences in gene expression. EGR2 has been related to $CD4^+$ T cell anergy (Safford et al. 2005). EGR1 is an immediate early gene induced quickly after antigen or chemokine activation of T and B cells (McMahon and Monroe 1996), and has been shown to limit TRAIL expression on NK cells (Fu et al. 2003; Balzarolo et al. 2013). Another interesting gene is **IRF8** (-4.29) that may be involved in the expression of genes related to cytotoxicity induced in response to IFNs.

- *Cytotoxicity*: Many genes related with NK cell cytotoxicity appeared downexpressed on ionomycin treated NK cells as: **Granzyme K** (-3.25), **Granzyme A** (-2.98), **Granzyme H** (-2.67), **Granulysin** (-2.97), **Cathepsin W** (-2.98), **Lymphotoxin B** (-2.63), **TNF-α** (-3.10). Changes in the expression of all these molecules will imply profound defects in NK cell cytotoxicity, and could explain why although some ionomycin treated NK cells were able to degranulate**(Fig. 3)**, they were completely

unable to kill K562 cells **(**Fig. 4**)**.

- *Extracellular Matrix*: Deficiency of **TIMP1** (-2.34), a matrix metalloproteinase inhibitor, and the metalloproteinase leukolysin**MMP25** (-2.20), could produce changes in the set of molecules exposed on the NK cell surface and how they interact with the extracellular matrix to affect adhesiveness, migration or ligand recognition.

- *Receptors*: **CD300A** (-4.24) and **CD300C** (-4.81) are the two most downregulated genes in our analysis. The CD300 family recognizes lipids and CD300a has been described as an inhibitory receptor (Lankry et al. 2013), whereas CD300c acts to activate NK cells (Borrego 2013). The Dap10 gene (**HCST**, -2.74) is an NK cell adaptor molecule important for the transmission of signals from the NKG2D receptor, and its deficiency may imply a decreased ability to signal through this receptor. In our redirected antibody cytotoxicity experiments we observed that after ionomycin treatment, lysis triggered by stimulation of the NKG2D receptor is more affected than activation through other receptors such as NKp46 **(**Fig. 22**)**. mRNA for the NKp30 receptor also scored as downmodulated (**NCR3**, -2.27), but no difference was observed at protein level. **CD27** (7.24) has been related to murine (Hayakawa and Smyth 2006) and human (Fu et al. 2011) NK cell developmental states, NK cell activation (Yang et al. 1996), reduction of the threshold for $CD8^+$ T cells responses (van Gisbergen et al. 2011), and chronic ligation of this receptor has also been linked to $CD8^+$T cell exhaustion (Tesselaar et al. 2003); however, although in some ionomycin treated cells increased cell surface expression of CD27 could be observed, this was not seen for all donors.

- **SPRY2**: Sprouty2 (-2.63) acts to inhibit receptor tyrosine kinase signaling proteins, decreasing PLCγ signals in T cells, blocking T cell receptor induced calcium release and limiting antigen receptor mediated activation (Akbulut et al. 2010), so that deficiency of SPRY2 may lead to an overbalance of this pathway. This gene has been reported to be specifically overexpressed in iNKT, γδT and NK cells (in comparison with other immune cells) which suggest it may have some special regulator function for these lineages (Bezman et al. 2012). It also sequesters c-Cbl inhibiting its effects on receptor downregulation (Wong et al. 2002).

- The dual specificity phosphatases **DUSP2**(2.72) and **DUSP4** (2.41) are nuclear members of the Dusp family which dephosphorylate threonine and tyrosine residues of MAP kinases p38, ERK or JNK. Due to their nuclear localization they can directly inhibit transcription factor activation. Dusp4 is the highest expressed member of the Dusp family expressed on NK cells (Jeffrey et al. 2007).

- MAL **(2.92)** and CAV1 **(4.06)** are both membrane proteins which appear in the internal detergent-insoluble microdomains and are involved in receptor clustering, receptor traficking and receptor activation in T cells (Millan et al. 1997; Anton et al. 2011; Tomassian et al. 2011), however they are expressed at only very low levels on NK cells. Overexpression of these molecules could modulate receptor clustering and cell activation.

5.2 Transcription factors and microRNAs associated with differentially expressed genes

Online tools like Genecodis, can also be used to predict microRNAs which could suppress our list of genes. The upregulated list of genes could be target of many different microRNAs where hsa-miR-516a-5p (*ramp1, cd109, uchl1, mal, vcam1, hmox1, igj, cd27,* kiaa1324) and hsa-miR-766 (*lrrc16b, ramp1, igfbp2, b3gnt7, creb3l3, itgb5,* kiaa1324) are the most significantly enriched. When the downregulated list of genes was examined, several appeared as possible targets for hsa-miR-106b* (*rgl4, apobec3h, c10orf128, snca, ltb, cd52, ccl3, ccl3l3*),hsa-miR-516b (*stom, ncr3, tspan32, ccr3, spry2, tnf, cd300a, irf8, egr1*) and hsa-miR-936 (*nt5e, ltb, gnly, gzma, styk1, ccl4l1, ccl4, egr1*), but many others appeared as regulators of 8-7 genes. However, when these analyses were repeated using a different program; g:Profiler, only miR-343 appeared to be significantly associated with the upregulated list (*reep2, hes6, lmna, plau, igfbp2, hoxb6, dusp4, nbl1, ramp1*) and no miRNA was associated with the downregulated genes. From these analyses no clear microRNA candidates appeared as mediators of the altered gene expression in the ionomycin treated cells

Using g:Profiler, we could associate also transcription factors associated with our genes. For the upregulated list, only **SP1** appeared as related with some of them (*cthrc1, cd9, epas1, cav1, tnfrsf9, cyp1b1, reep2, p2rx5, itgb5, hes6, fabp5, flvcr2, b3gnt7, rnf130, cbfa2t3, hspb1, mal, moxd1, rdh10, top1mt, dusp2, uchl1, igfbp2, tmem117, col6a1, magef1, bmf, slc7a5, ppap2a, cercam, dusp4, nbl1, prkcdbp, tubb2a, prkce, lrrc28, lrrc16b, pmepa1*) While **SRF** (*egr1, matk, egr2, ccr5, plac8, nt5e*) and **c-rel** *(irf8, matk, ccl4, xbp1, ptgdr, gzma, egr2, cd52, ncf2, gzmh, cxcr6, ccr3, tspan32, cxcr7, slamf8, mmp25,* ENST00000400702) appeared as candidates to be involved in regulating expression of molecules in the downmodulated list.

C-Rel is a member of the NF-κB family of transcription factors and its deficiency has been related to T cell tolerance and anergy susceptibility. It also appears not to be activated in immature B cells (Deenick et al. 2009; Liou and Smith 2010). In fact, anergy induction by ionomycin in $CD4^+$ T cells is thought to be driven by NFAT dimers, formed in the absence of c-Rel and AP-1, that cause the expression of anergy inducing genes, and the expression of proteins which could degrade c-Rel (Macian et al. 2002). C-Rel did not appear to be downmodulated after ionomcyin treatment of NK cells, but other proteins could be targeting it after ionomycin treatment and reducing its protein levels, leading to the observed lower expression of genes regulated by c-Rel (*(irf8, matk, ccl4, xbp1, ptgdr, gzma, egr2, cd52, ncf2, gzmh, cxcr6, ccr3, tspan32, cxcr7, slamf8, mmp25,* ENST00000400702*)*.

5.3 Comparison with data from the literature

To further understand the importance of the differentially expressed set of genes obtained in the ionomycin treated NK cells analysis, the gene signature obtained on these cells was compared with data from a range of articles where the mRNAs expressed by NK cells under different treatments or from tumor of virus infected patients had been characterized. The mRNA profile of ionomycin treated NK cells was also compared with those of other hyporesponsive cells such as anergic $CD4^+$ T cells or exhausted $CD8^+$ T cells.

However, there exist other studies where functional NK cell changes were not accompanied by significant changes in gene expression. For example, when the transcriptome of unlicensed NK cells isolated from mice deficient in MHC-I or ß-2 microglobulin genes and so hyporesponsive were analyzed (Guia et al. 2011) where different genes appeared differentially expressed, but the only gene in common between the different models of unlicensed NK cells was *Klra6* which encodes Ly49F, an inhibitory receptor expressed on murine NK cells, implying that, at least in mice, there was no transcriptional signature for this NK cell licensing model. Also NK cells isolated from the peripheral blood of human patients with melanoma showed no differential gene expression compared to NK cells purified from controls (Critchley-Thorne et al. 2007), although an important limitation to this study was that the functional capability of NK cells from these patients was not characterized.

The use of online tools, such as the EMBL-EBI Expression Atlas http://www.ebi.ac.uk/gxa/, or http://marq.cnb.csic.es/, which perform automatic data mining comparing gene sets with published data didn't identify significant similarity between any published signature and the lists of genes downregulated or upregulated in ionomycin-treated NK cells. Therefore a manual comparison of the changes in gene expression seen in ionomycin treated NK cells, with data from different papers was undertaken to find coincidences or differences between different signatures. These analyses were represented using Venn diagrams and are presented in **Appendix 1**. As genes can have different alternative denominations, to avoid the lost of coincident genes all list were translated to Entrez numbers. For these reason, only 69 of the 72 upregulated genes, and 72 of the downregulated 74 genes were used as the other 5 genes do not have an Entrez associated number.

There exist several articles analyzing NK cell gene expression signatures. In (Dybkaer et al. 2007) the authors compared the gene expression of human NK cells with the molecular signature of other cell types. Comparison of their gene analysis with the lists of genes altered by ionomycin, revealed that 3 ionomycin upregulated genes, appeared in their gene analysis, but, 18 of the genes which define their NK cell signature were downmodulated. These data are consistent with a possible NK-*dedifferentiation* process. Moreover, comparison of the ionomycin signature with those of activated $CD56^{dim}CD16^+$ NK vs.

CD56^{dim}CD16^{+} NK cells again identified multiple genes in common with the downregulatedionomycin signature (Hanna et al. 2004). The lists of ionomycin regulated genes were also compared with the signature described for human decidual NK cells (dNK) (Koopman et al. 2003) which is a special subtype of NK cells important in maternal-fetal tolerance. Surprisingly several upregulated genes in dNK cells were also upregulated in ionomycin treated cells, and there were also some genes found in common with the genes which have lower expression in ionomycin treated cells. Proteomics analysis of molecules enriched after stimulation of NK cells with IL-2 has also been published (Ma et al. 2013), and again molecules were found in common only between proteins upregulated by IL-2 and downregulated in ionomycin treated NK cells.

The molecular signature of murine NK cells has also been analyzed recently (Bezman et al. 2012). The signature described in this article is, not surprisingly, somewhat different from that described for human NK cells (Dybkaer et al. 2007), but again some genes important in this NK cell signature appeared as downmodulated in ionomycin treated cells, and none were found in common with those upregulated in the ionomycin treated cells. Moreover, in this article, the authors also analyzed the variation in the genes expressed by NK cells during the course of an MCMV infection. Few genes were found in common between the ionomycin-treated NK cells and the murine NK cells from different phases of MCMV infection, but for those that were identified, the ionomycin downmodulated signature coincided with genes of the early effector response, while the upregulated corresponded with genes modulated in the late and memory responses. These observations imply that the transcriptome of ionomycin treated NK cells may be related to the gene signature of NK cells in the later stages of a response, with perhaps a relative inability to mount a new effector response.

The gene signatures of ionomycin treated NK cells were also compared to the transcriptome profiles of NK cells isolated from human cancer patients. Comparison with the NK cell signature from IL-2 expanded NK cells *vs.* freshly isolated human NK cells from metastatic patients (Park et al. 2010) and with data of gene expression of NK cells from myeloma patients *vs.* control samples (unpublished GSE27838) also identified genes whose expression was modulated in cancer and after ionomycin.

Gene expression of ionomycin treated NK cells was also compared with the genes differentially expressed in different situations associated with hyporesponsivenes of CD4^{+} and CD8^{+} T cells (anergy, exhaustion, tolerance) and again some genes were found with similar or inverse regulation: studies with human non-tumor reactive CD8^{+} T cells *vs.* tumor reactive CD8^{+}T cells, tolerant murine CD8^{+}-T cells (Schietinger et al. 2012), tumour specific human CD8^{+}T cells infiltrating lymph nodes (TILN) *vs*. peripheral blood tumour infiltrating CD8^{+}T cells (Baitsch et al. 2011), mouse HIV exhausted CD8^{+} T cells (Quigley et al. 2010), mouse chronically infected with LCMVclone13 exhausted CD8^{+} T cells *vs*. effector CD8^{+}T cells (Wherry et al. 2007). Surprisingly no genes modulated by ionomycin in NK cells were found in common with the genes upregulated in ionomycin treated CD4^{+} T cells described by Macian *et al*. (Macian et al. 2002).

Although obviously speculative, overall these analyses suggest that ionomcyin treated NK cells have reduced expression of genes whose expression is associated with NK cell activation and differentiation for effector function which could underly their reduced ability to respond to target cells. Interestingly ionomycin treated NK cells also upregulate some of the genes which are upregulated in decidual NK cells, which are poorly cytotoxic, and instead exert regulatory functions (Kopcow et al. 2005; Hanna et al. 2006). Interestingly, the gene signature of ionomycin treated NK cells didn't correspond strongly with the signature from other hyporesponsive cells under different circumstances, thus although some genes were regulated in common no potential candidate was identified as a common gene which could regulate the responsiveness of both NK and T cells. The only upregulated gene which appeared in common between hyporesponsive T cells and ionomycin treated NK cells was *tnfrsf9* whose protein product is **4-1BB** (CD137), a member of the tumor necrosis factor. 4-1BB is expressed on the surface of human NK cells after activation, where its interaction with its ligand impairs cytotoxicity and IFN-γ production by human, but not murine, NK cells (Baessler et al. 2009; Buechele et al. 2012). Although ligation of 4-1BB does not affect the effector functions of murine NK ells it has been described to inhibit the development of these cells in mice (Choi et al. 2010). Inhibition by 4-1BB has been proposed to be mediated through TRAF3 and TRAF2 blockade of NF-κB activation (Barao 2012), but this has not been studied in detail.

Discussion

The role of the immune system in cancer; tumor-promoting or tumor-antagonizing is still a topic of active debate. Although the beneficial role of immunity in the defense from pathogens or tumor transformation is clearly demonstrated in immunocompromised individuals or in different models of carcinogen-induced tumors; the immune system response can also have a, paradoxical, tumor promoting effect by secreting growth factors, matrix modifying enzymes or reactive oxygen species; or by selecting antigen-escape variants and contributing to the immunoediting of tumor variants. Immune-surveillance and immune-editing are proposed to take place in three steps: elimination, equilibrium and escape that comprise a mechanism by which tumors and pathogens avoid, resist or suppress immune vigilance and eventually escape from immune control (Dunn et al. 2002). This evasion of the immune system has been proposed as an emerging hallmark of cancer (Hanahan and Weinberg 2011). Unresponsive NK cells have been characterized in patients suffering different types of tumor or different infections, however, as summarized in the introduction many different contradictory phenotypes have been described for these cells, due probably to the heterogeneity of diseases and models used; and little is known about the biochemical changes undergone by an NK cell that becomes unresponsive.

In this work we studied the use of ionomycin as a method to induce NK cell hyporesponsiveness, mimicking chronic NK cell stimulation or unbalanced stimulation due to an activating signal in the absence of inhibitory signalling. This model has the advantage of simplicity, reproducibility and as it is a non-specific stimulation, it reduces the likelihood of identifying effects associated with specific receptors and highlights common processes. Moreover since ionomycin treatment has been used previously to study anergy in $CD4^+$ T cells (Macian et al. 2002), we could use this information as guidelines to study how hyporesponsiveness was produced in NK cells. Release of calcium from intracellular stores and influx of extracellular calcium, is one of the first steps after target cell recognition, and correlates with exocytosis of the lytic granules and IFN-γ production (Bryceson et al. 2006), so the initial hypothesis was that mimicking this first influx would simulate chronic stimulation.

Ionomycin treatment resulted, in a dose and time dependent manner, in a reduced **ability** of IL-2 cultured NK cells **to degranulate** in response to K562 target cell stimulation **(**Fig. 1**)**. Optimum inhibition was obtained using 1μM ionomycin in an overnight treatment. This protocol is almost identical to that used previously to induce anergy in $CD4^+$ T cells. We decided to include, after washing and seeding in fresh media ionomycin treated cells, a 24 hour resting time with the idea that this would allow the ionomycin treated cells to reestablish their cell homeostasis. Carrying out the experiments in this way also ensured that the hyporesponsive state was stable for at least 24hours. Culture of the ionomycin treated cells for longer times was not carried out because of worries about the reduced viability of control and ionomycin treated cells cultured in the absence of IL-2. Interestingly, a constant level between donors of about 50%, of the ionomycin treated NK cell population was observed to still be able to degranulate in response to target cells. Moreover, ionomycin treatment produced elevated levels of cell death for NK cell lines from some donors, while NK cells from other donors, were relatively unaf-

fected and indeed needed a higher dose of ionomycin to induce unresponsiveness. These results suggested the hypothesis that ionomycin could be tuning NK cells responsiveness, and the efficacy of this treatment would vary depending on the donor. Moreover, if each NK cell clone differs in susceptibility to ionomycin and in response to stimulation, there would exist in the NK cell population of any given individual NK cell clones more sensitive, and more resistant, to the ionomycin-mediated readjustment.

The ability of ionomycin treated NK cells to kill target cells other than K562 was also studied, and although the levels of reduction in response varied, ionomycin treatment consistently led to decreased NK cell degranulation against the panel of targets tested **(**Fig. 3**)**. This defect in target cell recognition was further evaluated by assay of target cell lysis and ionomycin treated NK cells displayed a marked **inability to kill** K562 and Jurkat target cells **(**Fig. 4**)**.

No differences in the expression of activating or inhibitory NK receptors were found when control and ionomycin treated NK cells were compared by flow cytometry, indicating that ionomycin induced loss of NK cell function was not due to defects in the NK cell receptor balance, **however lower levels of integrins** (mainly αL, αM and ß2 chain) were expressed by the ionomycin treated cells **(**Fig. 13 & Fig. 14**)**. Interestingly, treatment with IL-2, that restored NK cell degranulation led to the recovery of expression of various integrin except for the β2 chain **(**Fig. 14-C**)**. The distribution of a range of different receptors among the NK cell populations that do, or do not, degranulate after exposed to K562 was also analyzed, and again no differences were found between them **(**Fig. 18**)**, thus, it could be discarded that in ionomycin treated cells the NK cell repertoire was shaped toward the selection of NK cells with impaired cytolytic activity. These data also argue against the idea that the population of cells inactivated by ionomycin treatment represents a particular subset of NK cells. Modulation of expression of different adaptor molecules has also been described in NK cells after chronic stimulation with target cells (Coudert et al. 2005), and in NK cells isolated from tumor patients (Lai et al. 1996) and a **decrease in CD3-**ζ chain **expression was noted in the ionomycin treated NK cells**. Given that many activating NK receptors can associate with either CD3-ζ or the FcεRγ chain (Vivier et al. 2004), this downmodulation of adaptor molecules together with the maintenance of receptor expression may imply changes in the usage of adaptor molecules. Degradation of adaptor molecules has been described previously in T and B lymphocytes after TCR or BCR stimulation (Kishimoto et al. 1995; Vilen et al. 1999; Vilen et al. 2002; La Gruta et al. 2004) or after NKG2D chronic stimulation in T cells (Hanaoka et al. 2010). Moreover, the recruitment of FcεRγI to the TCR complex, instead of CD3-ζ has been described in T cells from patients suffering *Systemic lupus erythematosus* (Enyedy et al. 2001) and in T cells from tumour bearing mice (Mizoguchi et al. 1992). Although, the significance of this FcεRγI-CD3ζ exchange of the TCR is not clear (Frey and Monu 2008), the use of different adaptor molecules implies important changes in cell signaling, as FcεRγI has a higher affinity for Syk than for ZAP-70 leading to different kinetics in signal transmission (Paolini et al. 1995), moreover CD3-ζ expresses three ITAMs whereas FcεRγI has only one. The consequences of specific CD3-ζ degradation for NK cell function are unknown, but a population of NK cells deficient for FcεRγI in normal individu-

als has been described. These γ-deficient NK cells expressed lower levels of NCRs and were poorly responsive to tumour target cells, but were highly effective in mediating ADCC (Hwang et al. 2012). These cells have also been reported to express a different repertoire of KIR receptors, and the authors proposed that they could represent uneducated or unlicensed NK cells that although able to mediate ADCC, are unable to respond to other stimuli. By analogy, CD3-ζ degradation could imply lower responsiveness either generally, or via specific receptors, but no consistent differences in activating NK receptor expression were noted in the inomycin treated NK cell population.

The lower level of expression of integrin molecules might possibly be related to the observation of **a lower efficiency in the formation of conjugates** between NK cells and K562 target cells **(**Fig. 10**).** Defects in conjugate formation have been described in NK cells from cancer patients (Peracoli et al. 1999; Montelli et al. 2001). Similarly, cord blood NK cells that also show impaired cytolytic ability, are unable to form stable conjugates and express normal levels of various receptor, except for LFA-1 and CD2. IL-2 stimulation restored these defects, moreover it was demonstrated that blocking of LFA-1 and/or CD2 resulted in profound inhibition of F-actin polarization and cytotoxicity, highlighting the importance of these molecules for NK cell cytotoxicity (Xing et al. 2010). β2 integrin-deficient murine NK cells display a hyporesponsive phenotype in vitro, perhaps related to alterations in their development and differentiation so that β2 integrins are required for optimal NK-cell maturation, but this deficiency is partial and can be bypassed during MCMV infection (Crozat et al. 2011). These data are consistent with the hypothesis that the reduced expression of adhesion molecules could play some role in NK hyporesponsiveness. However other data argue against this idea. In our hands, LFA-1 blocking of control cells, did not impair NK cell cytotoxicity (data not shown), perhaps because in these experiments long term IL-2 cultured NK cells were used as effectors. The ability of ionomycin treated cells to degranulate when stimulated through the CD16 receptor, is also consistent with the idea that the defect in integrin expression of the ionomycin treated NK cells, was not sufficient to explain defective conjugate formation by these cells. Finally, integrins have been shown to have an important role in polarization of the cytotoxic granules of NK cells (Bryceson et al. 2010), and although many ionomycin treated NK cells did not adhere stably to target cells, those cells that did form conjugates were able to polarize lytic granules and LFA-1 **(**Fig. 11 & Fig. 12**)**. Perhaps, because although lower, integrin levels continued quite high **(**Fig. 10**)** so ameliorating any possible effects associated with the integrin downmodulation in ionomycin treated cells. Interestingly, a reduced ability to form conjugates with target cells has been described in unlicensed human unlicensed NK cells (Thomas et al. 2013), and this defect has been attributed to a defect in inside-out activation of LFA-1. Moreover, lytic granule polarization also occurred normally on these cells. Thus, ionomycin treatment could be targeting inside-out activation, as we observed a decreased level of staining using the NKL-L16 antibody **(**Fig. 14-B**)**, but it could also be affecting a step involved in the release of lytic granules following their polarization.

The knowledge that ionomycin treated NK cells expressed the same levels of activating receptors

as control cells, allowed evaluation of the ability of NK cells to respond through different activating receptors by using redirected antibody mediated cytotoxicity assays. This approach allowed measurement of NK cell responses to specific stimulation of single receptors, and also to test whether combinations of receptor stimulations produced synergistic signals that might overcome ionomycin restriction. Ionomycin treated NK cells were able to **degranulate as efficiently as control cells when stimulated through the CD16 FcgRIII-receptor**, which is also the receptor that induces the most potent Ca^{2+} influx. Surprisingly, when the NK cells were triggered using pairs of antibodies specific for receptors known to synergize, although both control and ionomycin treated cells showed higher degranulation, these synergistic stimulations **weren't able to overcome the effect of the ionomycin treatment (**Fig. 19**)**. Interestingly, the ability to respond through Fc-receptors, but not NCRs, has been described as maintained in NK cells from HIV infected individuals (Mavilio et al. 2003).

Normal degranulation and IFN-γ secretion was **re-established following a 24 hour IL-2 treatment**, implying that the defect was reversible **(**Fig.5 & 7**)**. This IL-2 driven recovery of function is also a feature of other models of induced NK cell unresponsiveness (Abrams and Brahmi 1986; Tripathy et al. 2008) and has been observed with NK cells recovered from cancer patients (LeFever and Funahashi 1991; Konjevic et al. 2007; Reiners et al. 2013). These observations suggested that ionomycin induced hyporesponsive NK cells might still respond to inflammatory stimuli. However, IFN-α or IL-12 plus IL-18 stimulation did not lead to the recovery of the degranulation **(**Fig. 6**)**. When they were stimulated with IL-12 and IL-18, hyporesponsive NK cells produced much less IFN-g than untreated NK cells and while the production of IFN-g by the control NK cells was further increased on exposure to target cells, ionomycin treated NK cells did not produce more IFN-g after stimulation with target cells. **(**Fig. 7**)**. Analyses for possible defects in IL-12 and IL-18 receptor expression did not identify any differences between control and ionomycin treated cells **(**Fig. 16**)**.

Experiments done with **freshly isolated** NK cells, showed that **ionomycin is able to induce a hyporesponsive state** in these cells similar to that observed for ionomycin treated activated NK cells, however, some differences were observed. Interestingly, **IL-2 treatment was only partially able to restore the functionality of hyporesponsive fNK cells (**Fig. 24**)**. One possible explanation for this discrepancy is that IL-2 restored functionality is due to the activation of parallel activation pathways that act to compensate, but not restore, the ionomycin induced specific defect, and that this compensation can recover the function of activated NK cells. but not for fNK cells. Further experiments will be necessary to distinguish between these possibilities, but these differences between activated and freshly isolated NK cells, can help to understand the controversy found in the literature related to IL-2 driven recovery of hyporesponsive NK cells.

As discussed in the introduction, a threshold for NK cell responsiveness is established during their licensing or education. This setpoint is not fixed, but rather is a plastic process since mature NK cells are able to be re-educated during their entire lifetime. These previous data and the results from iono-

mycin treated NK cells suggest that activating signals and ionomycin could be shifting the NK cell activation threshold, so that while some cells maintain the ability to respond to target cell stimulation, form stable conjugates and polarize molecules to the immune synapse, after ionomycin treatment; another part of the population is rendered unresponsive, but able to respond when enough stimulation is received, as with CD16 stimulation. This hypothesis of variation in thresholds of responsiveness for activation would also explain why NK cells from some donors were insensible to 1µM ionomycin treatment, whereas others died due to the stimulation. As NK cell licensing is a flexible process, ionomycin could be resetting the responsiveness of each NK cell, re-tuning them and increasing the intensity of the signals necessary to mount and effective response.

This model is a logical extension of the disarming and rheostat models proposed for NK cell licensing. NK cell education is thought to occur in the bone marrow by interactions between precursor NK cells and stromal cells. The disarming model proposes that NK cells are generated as highly cytotoxic cells that need to be rescued from overstimulation induced unresponsiveness in a process of education, or licensing, that depends on the signal delivered by inhibitory receptors counterbalancing the activating signal (in the context of NK cell education this positive signal is likely to come from, as yet unidentified, endogenous activating ligands expressed in the bone marrow), **(Fig. 27-A)** (Wu and Raulet 1997). It is probable that this requirement for responsiveness to an inhibitory signal results in the generation of a more efficient activating circuit (Anfossi et al. 2006). The rheostat theory suggests that what guides NK cell education is the net balance of signals between activating and inhibitory inputs. This "balanced tuning input", defines for each NK cell a threshold for response, that will be related to the different intensities of responses made by different NK cells and influenced by the receptors expressed. This balance could be continuously re-evaluated throughout the NK cell's life. This way, the NK cell responsiveness will be like a rheostat, tuned depending on the balance between activating and inhibitory signals **(Fig. 27-B)** (Joncker et al. 2009). The disarming model can be extended to understand changes in responsiveness of a population of mature NK cells. When the balance between activating and inhibitory signals is below the threshold for response set for each NK cell, the cell would not respond. If the threshold is exceeded, the NK cell becomes activated. However, if the net balance is much higher, as could be happening in the contest of chronic activating receptor stimulation, a loss of responsiveness is induced in mature NK cells (Raulet and Vance 2006). NK cells would have then two thresholds, one for response, and one for inactivation due to overstimulation **(Fig. 27-C)**. The repertoire of activating and inhibitory receptors is determined stochastically for each NK cell clone so these checkpoints are likely to be specific for each clone and can also vary *eg.* after exposure to cytokines. Thus in a population of NK cells, cells would be present with a spectrum of different sensibilities to initiate a response. If the signal received exceeded the second threshold, NK cell clones would begin to become inactivated (disarming model), or the responsiveness could be readjusted downward until it is counterbalanced again, setting a higher threshold for response (rheostat model).

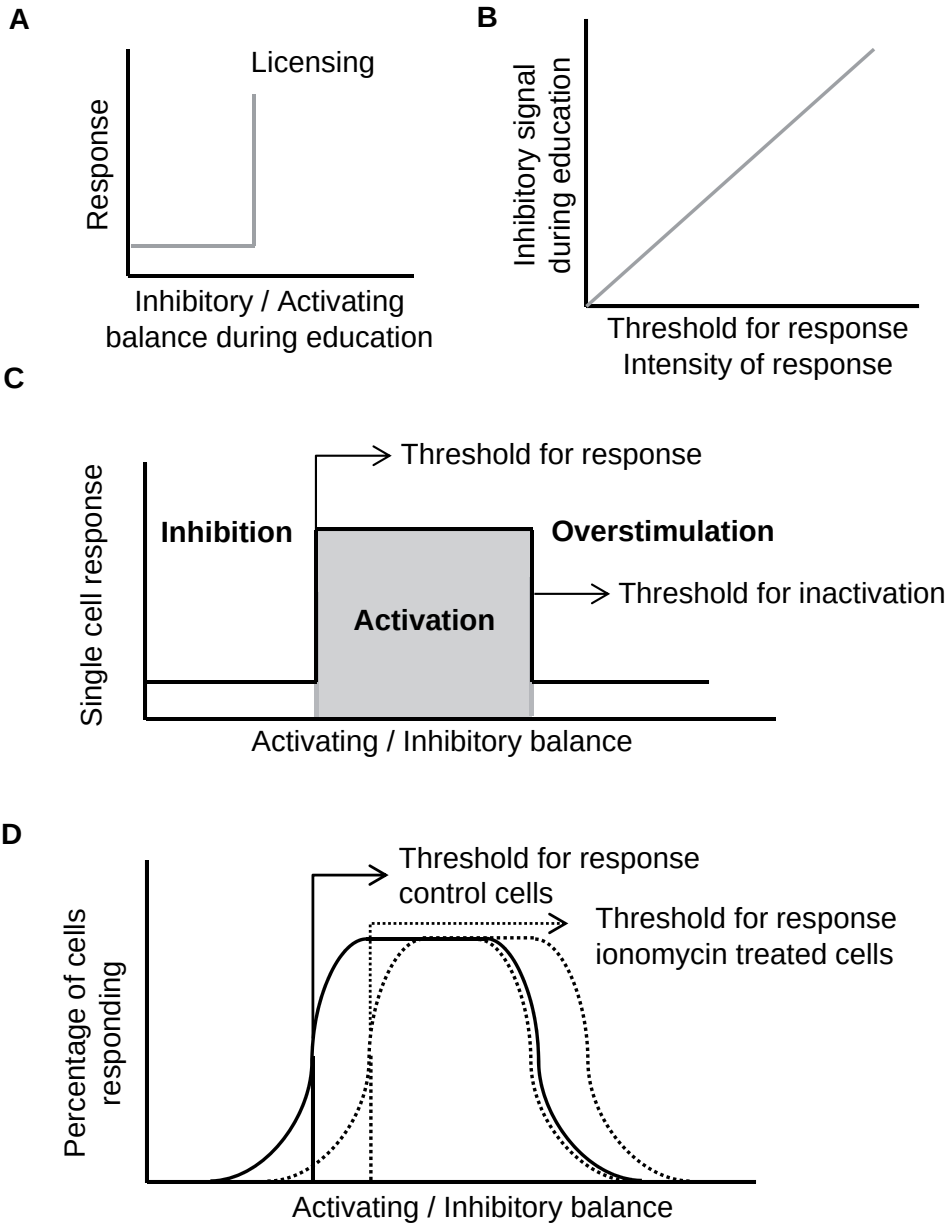


Fig. 27. (A) The disarming model proposes that the education of each individual NK cell depends on the balance of signals received from inhibitory receptors and activating receptors. **(B)** The rheostat model suggests that the threshold for response and the intensity of the response of each individual NK cell is fixed by the inhibitory signal received during education. **(C)** The disarming model also implies that during education there exists one threshold for inactivation, which if exceeded triggers NK cell hyporesponsiveness. When licensed, NK cells will have two thresholds: one for activation and another for inactivation after overstimulation. **(D)** Ionomycin treatment could cause a recalibration of at least the NK cell threshold for response, increasing the amount of activating signal required for an NK cell response.

Ionomycin treatment of NK cells, represents an overstimulation signal, which instead of causing higher responses of an NK cell population, recalibrates each NK cell rheostat establishing a higher threshold on them, so that the NK cell population becomes hyporesponsive or tolerant for stimuli which are able to activate control cells. Whether the upper threshold is also retuned is unknown **(**Fig. 27-D**)**. As the NK cell population consists of cells expressing various combinations of inhibitory receptors with different tuned thresholds, the ionomycin treatment would affect them differentially, so that while cells would be rendered hyporesponsive, others would still be able to degranulate despite the threshold recalibration

This process might be working when NK cells sense that they are being overstimulated, allowing the readaptation their sensitiveness and stopping target cell killing which could cause severe damages for the organism, but conserving at the same time their killing potential. This way when later they were exposed to more potent stimuli, they would still be able to respond efficiently. The "downside" of this mechanism may occur in the case of infection or tumour transformation, where infected or transformed cells could be exploiting this "fail-safe" mechanism against exacerbated responses from NK cells to escape from immune surveillance.

There are many questions still unanswered in this model: Does the chronic stimulation need to be potent to cause unresponsiveness? Or would a low intensity, but chronic, stimulation also be sufficient for inactivation if maintained enough time? Are chronic activation signals modifying how inhibitory signals are sensed, shaping the balance to a higher threshold?

NK cell threshold is established in a narrow range, and very small changes in MHC class I expression provoke sharp transitions of a cell from resistant to susceptible to NK cell lysis (Almeida and Davis 2006; Holmes et al. 2010). In this way, small changes in the levels of expression of activating or inhibitory ligands, would profoundly affect target cell susceptibility to NK cell attack. This tight control of NK cell reactivity acts to increase the sensitivity of the system and to prevent autoimmunity. Indeed it is very interesting that NK cells from distinct organs including the dermis, the gut, the lungs or the liver, which are constantly exposed to bacteria or toxins, show reduced cytotoxicity when compared to peripheral NK cells and NK cells isolated from pancreas, joints or brain. Similarly, decidual NK cells that are in contact with fetal cells also show reduced cytotoxicity (Shi et al. 2011). The maintenance of immunotolerance of organ infiltrating immune cells to this constant contact with signals that don't represent danger, while at the same time conserving the ability of these leukocytes to be ready to exert a fast response when necessary must require very efficient regulatory mechanisms (Sun et al. 2013). Constant expression in these tissues of NK cell ligands, could set the sensitivity of resident/infiltrating NK cells to respond while preventing nonspecific activation. One possible explanation for these organ-specific differences could be that the liver, gut or lungs are seeded with NK progenitor cells that generate mature NK cells

with characteristics different from peripheral blood NK cells (Chinen et al. 2007; Wu et al. 2012), for example the NK-22 cells that are found enriched in mucosal tissues including dermis, Peyer's patches or tonsils, whose generation is dependent on the microflora. NK-22 cells lack cytotoxic proteins and IFN-γ, but express RORγt and secrete other cytokines such as IL-22, and function to help to constrain inflammation (Satoh-Takayama et al. 2008; Cella et al. 2009). Another possibility for the diversity of organ-resident NK cells and their basal hyporesponsiveness, is that the microenvironment and the characteristics of these tissues are able to shape and modulate the population of mature NK cells that arrive to them. For example, liver NK cells are poorly cytolytic and although they express normal levels of receptors and cytotoxic proteins, they display decreased levels of inhibitory receptors for self-MHC class I. The liver is an organ continuously exposed to toxins, and it could be that the lower levels of MHC class I on hepatocytes and the continuous stimulation of NK cells, together with the liver microenvironment, change the NK cell threshold preventing autoimmunity (Burt et al. 2009). In contrast, lung derived NK cells express higher levels of inhibitory receptors and lower levels of activating receptors and adhesion molecules compared to peripheral NK cells, creating an altered threshold for response (Wang et al. 2012).

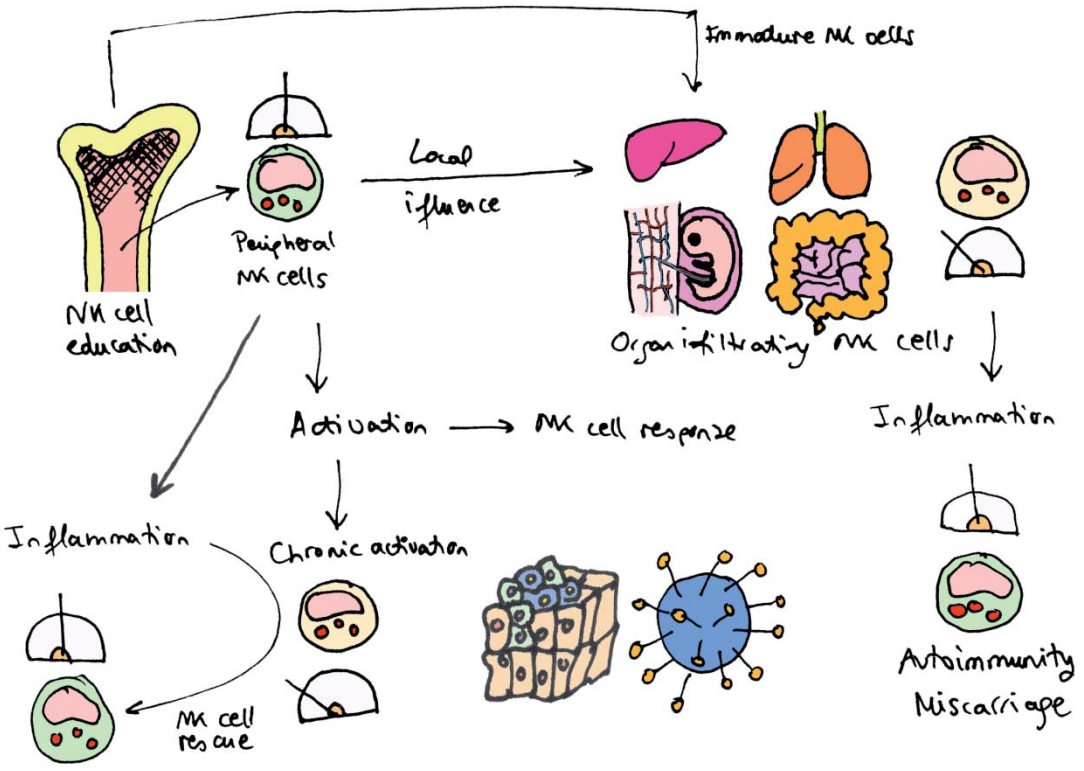


Fig. 28. Schema comparing organ infiltrating NK cells and hyporesponsive NK cells threshold. NK cells infiltrating different organs are described to be hyporesponsive. Whether they represent NK cells from different origins or peripheral NK cells adapted to their environment is still being discussed. Peripheral NK cells could also be adapted to their milieu during chronic activation, existing a parallelism between organ infiltrating and chronically activated NK cells.

A tunable signaling threshold allows a better interpretation of the context of ligand presentation and therefore allows the generation of an immune response or the activation of tolerogenic programs depending on the circumstances. This equilibrium could be altered by the inflammatory stimulation, and the secretion of different cytokines, chemokines or antibodies, or the interaction with other cells, could lower the NK cell threshold for response, favouring NK cell activation. For example, in the presence of IL-2 (Burt et al. 2009), or in contact with Kupffer cells (Tu et al. 2008) liver NK cells are able to degranulate as efficiently as peripheral NK cells; and even the NK-22 population changes its IL-22 production towards IFN-γ production when stimulated with IL-2 (Cella et al. 2010). However, if this response becomes uncontrolled, it could give rise to immunopathologies (Li et al. 2012) **(Fig. 28).**

Little is known about how the threshold for NK responsiveness is maintained and at the same time can be re-evaluated and varied. Microarray data with NK cells from different mice deficient in MHC class I, and thus with NK cells unlicensed or tolerant, didn't identify any change in gene expression that correlated with activation state, being the gene of Ly49F the only one commonly overexpressed. However, this study demonstrated that NK cell activating receptors in unlicensed NK were confined together with inhibitory receptors in actin meshwork structures, in contrast to the nanodomain structures where they localize in educated NK cells and move more freely (Guia et al. 2011), suggesting that the lack of signaling coming from KIR receptors could be sufficient to alter the spatial localization of activating receptors in the membrane, without further need for differential gene expression. Recently it has also been shown that an activating signal modifies clustering of inhibitory receptors (Pageon et al. 2013), establishing that inhibitory and activating signals can affect how NK cells are going to respond to posterior signals via alteration of receptor movement in the membrane. After these two studies, the question that remains still unanswered is, could chronic activating signal differentially affect the localization of inhibitory and/or activating receptors? Compartmentalization of membrane receptors and signaling molecules in regions of the cell membrane is very important for the initiation of cell activation and the regulation of signaling. Disorganization of immune receptor localization would affect not only the recruitment of molecules to the immune synapse, but could also affect the potency of the signal generated, make more difficult the establishment of synergies between different activating receptors and might also increase the ability of the inhibitory signal to block the action of activating receptors. The balance of activating *vs*. inhibitory signaling is established not only by a one to one interaction, but also, by how these molecules are clustered and organized, as clustering will facilitate downstream signaling by the proximity of signaling molecules; so small changes in the spatial organization of receptors and signaling molecules could profoundly affect cell activation.

A number of possible factors could influence protein organization in the cell membrane, including lipid or sugar post-translational modifications, anchoring or tethering of proteins to actin filaments to create platforms that are transported together by actin polymerization changes. Alterations in these protein modifications or in the attachment of proteins to actin, caould affect NK cell function.

Acylation of proteins, by prenylation, palmitoylation, myristoylation or GPI modification, can mediate receptor inclusion in lipid rafts and so change their behaviour in the plasma membrane. A small number of proteins, such as signaling proteins like LAT or Src family kinases as well as GPI linked CD16, are found constitutively in lipid rafts, but others, including "Multichain immune recognition receptors" such as TCR, BCR or NCRs, become raft associated when they undergo transient lipid modifications. These receptors have been proposed to exist in an equilibrium; associating transiently with rafts in resting cells signaling for tonic signals, but when these receptors become activated, they oligomerize, move massively into rafts and start the transmission of signals by raft associated signaling proteins. Other proteins will then be recruited into rafts, the raft platforms will become larger by clustering and attach to the actin cytoskeleton stabilizing the platform and allowing the formation of an immune synapse. This segregation between raft and non-raft proteins allows the initiation, maintenance and prolongation of signaling. Different patterns of raft compartmentalization have been described in pre-T cells, in Th1 *vs*. Th2 subsets and in naïve *vs*. effector T cells highlighting the importance of raft organization for the outcome of the response (Dykstra et al. 2003). ZAP-70 has been shown to be excluded from rafts, by hyperactivation of Fyn, in anergic $CD4^+$ T cells (Lee et al. 2012) further suggesting a role for membrane segregation in anergy. In this context it is interesting to note that different types of lipid rafts, with different protein composition, have been described to allow spatial segregation of different signaling proteins, which came close only when the cell become activated (Filipp et al. 2012). Analysis of the protein composition of lipid rafts and their localization after activation on control cells compared to ionomycin treated NK cells could help to better understand NK cell hyporesponsiveness.

Glycoproteins have also been proposed to exert important roles in tuning immune signal thresholds by, for example, favouring the association of receptors with other glycoproteins, limiting receptor clustering or preventing receptor endocytosis (Rabinovich and Croci 2012). In the case of the TCR it has been proposed that its glycosylation restricts lateral movement of the receptor, increasing the T cell threshold for response, whereas defects in the GnT5 glucosyltransferase lower the threshold, and can predispose to autoimmunity. Galectin-3 has also shown to be able to trap the TCR after chronic stimulation of $CD8^+$T cells, distancing the TCR from the CD8 costimulatory molecule and creating an anergic state (Demotte et al. 2008).

Many transmembrane and cytosolic proteins can interact either directly or through adaptor molecules with ezrin/radixin/moexin (ERM) proteins, anchoring them to the **actin cytoskeleton** and coordinating their movements. Dephosphorylation of ERM proteins is thought to be essential to increase cell deformability allowing cell-cell contacts and facilitating immune synapse generation. Dephosphorylation also allows the detachment of receptors from ERM proteins enabling their relocation to different zones of the immune synapse (Ivetic and Ridley 2004). Changes in proteins involved in actin-anchoring have already been related to changes in NK cell responsiveness, for example Kindlin-3 whose deficiency in LAD-3 patients caused a large decrease in the response to single receptor stimulation, but high levels

of ligands or target cell mediated multiple receptor stimulation can cause activation similar to control cells, suggesting changes in the NK cell threshold (Gruda et al. 2012).

Further analysis of the importance of these modifications, and the localization or receptors in specific regions of the NK cell membrane, could provide clues that lead to a better understanding of NK cell activation and responsiveness. As it has been described previously the sequestering of inhibitory receptors in actin meshworks in uneducated NK cells (Guia et al. 2011), we analyzed the effect of the inhibition of actin polymerization on ionomycin treated NK cells by studying the Ca^{2+} flux triggered by antibody stimulation of a synergic combination of receptors. Surprisingly, actin polymerization seems to constrain NK cell responsiveness in both, control and inomycin treated cells **(**Fig. 21-C**)**, and although latrunculin treated hyporesponsive cells achieve to generate similar responses to DMSO control cells without disruption of their microfilament organization, latrunculin treatment of them increases also in our hands the responsiveness of control responsive cells.

With the aim of identifying factors important for hyporesponsiveness setting and maintenance the transcriptomes of control and ionomycin-treated NK cells were compared using cDNA microarrays, obtaining a signature comprising **75 upregulated and 72 downregulated genes**. Comparison of these genes with transcription profiles associated with different NK cell states and hyporesponsive lymphocytes of other lineages highlighted few genes in common **(**Annex 1**)**. However, it was surprising to find that many of the genes specifically upregulated on the NK cell linage, appeared as downmodulated in hyporesponsive cells perhaps indicating a loss of the “natural killerness” of the ionomycin-treated NK cells. As described in the introduction, lower expression of genes important for NK cell effector function, including granzyme A and B (Al Omar et al. 2011) or perforin (Bajpai et al. 1991), has been described in NK cells from patients suffering different types of cancer.

Further, some of the genes upregulated after exposure to ionomycin appeared in common with decidual NK cells (dNK) and NK-22 cells. Human dNK cells express normal levels of lytic granules and activating receptors, and are able to polarize LFA-1 and F-actin to the immune synapse, but not their cytotoxic granules, thus they are poorly cytotoxic and instead act as immunoregulatory and angiogenic cells (Kopcow et al. 2005). However, these cells are able to exert effector functions in the case of viral infections during pregnancy (Siewiera et al. 2013). It seems reasonable that a more detailed analysis of the genes expressed in common between ionomycin treated cells and dNK could give some clues about NK cell hyporesponsiveness. Incubation of peripheral NK cells with TGF-ß, an inhibitory cytokine produced by many tumours, has been shown to induce a state similar to dNKs (Keskin et al. 2007). This may indicate that chronic stimulation, as may happen in tumors, can lead to changes in NK cells similar to that suffered by dNKs to avoid maternal immune rejection during pregnancy. In fact, a process of “decidualization” has already been proposed to have occurred in NK cells purified from cancer patients (Belisle et al. 2007; Holtan et al. 2011). Another possibility is that the chronic stimulation together with the tumor microenvironment may be inducing a dediferentiation process in NK cells. It has been proposed that tumors were

colonized by NK cells in an early developmental stage, as judged by their phenotype, which were then rendered tolerant to the tumor (Carrega et al. 2008; Jin et al. 2013). However another way of interpreting these data could be that the tumor may be promoting a loss of maturation of mature NK cells together with their conversion into a regulatory phenotype.

It would be interesting to identify genes whose expression could explain the differential spatial localization of receptors in ionomycin treated NK cells. In the cDNA microarray studies of the effect of ionomycin, a number of genes related with post-translational modifications of proteins in the membrane were found, such as CTHRC1 (which is involved with N-glycosylation), heparanase (degrades polymeric heparan sulfate molecules) or urokinase (activates extracellular matrix degradation). Genes related to actin polymerization, incuding *pls3* which codifies for Plastin-3 or Fimbrin (which crosslinks actin filaments and also stabilizes actin dynamics without the needing of crosslinking, and has roles in integrin signaling were also identified. Interestingly, pls3 is a protein whose expression and activity is dependent on Ca^{2+} (Samstag et al. 2003; Begue et al. 2012). Future analysis of the role of these proteins in NK cells, and the consequences of their inhibition or overexpression may help to understand how hyporesponsiveness is established on NK cells.

Interestingly the largest group of genes downmodulated by exposure to ionomycin were chemokine receptors and ligands. This deregulation together with the lower expression of integrin molecules could cause defects in NK migration and tissue infiltration. Moreover, loss of the ability to detect chemokines will affect integrin activation, and as signaling through chemokines and cytokines are very important for NK cell priming, could also affect NK cell activation. Considerations such as these might help to explain the poor NK cell infiltration in malignant lung tumours, in contrast to non-malignant tumours (Esendagli et al. 2008). Surprisingly, the expression of genes coding for proteins such as E3-ubiquitin ligases (Mueller 2004) or caspase-3 (Heissmeyer et al. 2004) which are known to be upregulated in anergic T cells or after chronic stimulation through NKG2D in T cells (Hanaoka et al. 2010), and could potentially modulate the NK responsiveness by degrading signaling components was not found to be altered in ionomycin treated NK cells.

This review of data in the literature, together with the idea that the threshold for response of NK cells coming from different organs could be modified by constant exposure to activating signals or the microenvironment, are consistent with the idea that tumors or pathogens might take advantage of the tight control mechanisms that regulate NK cells, to prevent autoimmunity, and render them tumor-tolerant/infection-tolerant NK cells.

Ionomycin treatment has shown to be a useful tool to understand NK cell unresponsiveness and this thesis reports a detailed characterization of the phenotype and function of these ionomycin-induced hyporesponsive NK cells. However while multiple mechanisms that could possibly underly the hyporesponsive state have been tested, to date the molecular basis of the lack of response remains unclear. However the integration of the data described in this thesis to derive a plausible model for NK hyporesponsiveness should aid further understanding of this phenomenon by providing a conceptual framework that will be useful to generate hypotheses to guide future analyses of the properties of hyporesponsive NK and investigations into the bases of NK cell unresponsiveness.

Conclusions
Conclusiones

1. The treatment of activated NK cells with ionomcyin induces a loss of function in a time and dose dependant manner, being the optimal treatment being a 1μM dose of ionomycin for 16 hours in the absence of IL-2.

2. Activated NK cells treated with ionomycin are less able to form conjugates with target cells and to degranulate. They are also unable to secrete IFN-γ efficiently in response to target cells, even after stimulation with IL-12 and IL18. However, those ionomycin treated cells that were able to create conjugates with target cells, were fully able to polarize their cytotoxic granules, LFA-1 and F-actin. Stimulation of activated NK cells treated with ionomycin with IL-2 restores their functionality.

3. The expression of the αL, αM and β2 integrins is reduced in activated NK cells treated with ionomycin, although the expression of activating and inhibitory receptors is normal. The responses of activated NK cells treated with ionomycin were dimished after mAb mediated stimulation of specific receptors, with the exception of CD16 stimulation which triggered responses comparable to those observed in control NK cells.

4. The transcriptomic analysis of activated NK cells treated with ionomycin revealed the differential expression of 75 downregulated and 72 upregulated genes. Comparison of these altered patterns of transcription with transcriptional signatures of other classes of hyporesponsive cells showed that the hyporesponsive status induced with ionomycin on activated NK cells represents a state different to those previously defined in other hyporesponsive lymphocytes.

5. Treatment of freshly isolated NK cells from healthy donors with ionomycin induces a hyporesponsive state similar to that observed after the treatment of activated NK cells. However, fNK cells did not recover their ability to degranulate and produce IFN-γ entirely in the presence of IL-2, and are unable to exert degranulation as efficiently as control cells when stimulated through CD16.

1. El tratamiento de las células NK activadas con ionomicina induce una pérdida de función de manera dependiente de la dosis y el tiempo de tratamiento, siendo el tratamiento óptimo para inducir un estado de menor respuesta el generado con una dosis 1µM de ionomicina durante 16 horas en ausencia de IL-2.

2. Las células NK activadas tratadas con ionomicina muestran menor capacidad de conjugar con células dianas y de degranular, así como de secretar IFN-γ en respuesta a células diana incluso tras haber sido estimuladas con IL-12 y IL18. Sin embargo, aquellas células tratadas con ionomicina capaces de formar conjugados, son capaces de polarizar sus gránulos citotóxicos, LFA-1 y F-actina de manera efectiva. La estimulación con IL-2 es capaz de recuperar la función de las células NK activadas tratadas con ionomicina.

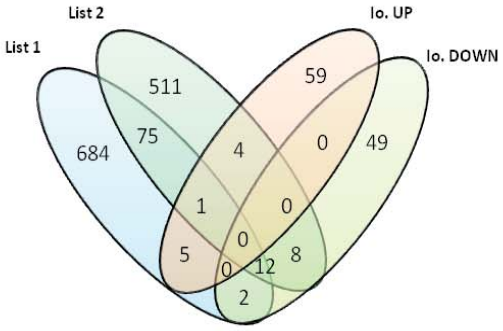
3. La expresión de las integrinas αL, αM y β2 se encuentra reducida en las células NK activadas tratadas con ionomicina, manteniéndose normal la expresión de los receptores activadores e inhibidores. La estimulación específica a través de los receptores activadores genera respuestas menores en las células NK activadas tratadas con ionomicina, excepto en el caso de la estimulación a través de CD16, que da lugar al mismo nivel de respuesta que en las células control.

4. Análisis del transcriptoma de las células NK activadas con ionomicina muestra que estas células expresan niveles reducidos de 75 genes y aumentados de 72 genes. La comparación de estos cambios en expresión génica con firmas transcripcionales de otros modelos de células que han perdido la función efectora, recalcó que el tratamiento con ionomicina de las células NK activadas genera un estado diferente al encontrado en otras células linfoides.

5. El tratamiento de células NK recién aisladas de donantes sanos con ionomicina induce una pérdida de respuesta comparable al observado con el tratamiento de las células NK activadas. Sin embargo, estas células no recuperan totalmente su capacidad de degranular y de secretar IFN-γ en presencia de IL-2; ni son capaces de responder a la estimulación a través de CD16 de manera tan eficiente como las células recién aisladas control.

Annex

1.- Gene expression of human NK cells compared with other cell types



Common elements in "List 1", "List 2" and "Io. DOWN":

GZMH	ITGB2
IL18R1	MYOM2
PLAC8	CTSW
GNLY	MATK
ITGAM	RGS18
CD300A	HCST

Common elements in "List 2" and "Io. DOWN":

CCL4	NCAM1
ADRB2	PTGDR
IRF8	NCR3
EGR2	TNF

Common elements in "List 1" and "Io. DOWN":
GZMA
SLAMF8

Common elements in "List 1", "List 2" and "Io. UP":
RAMP1

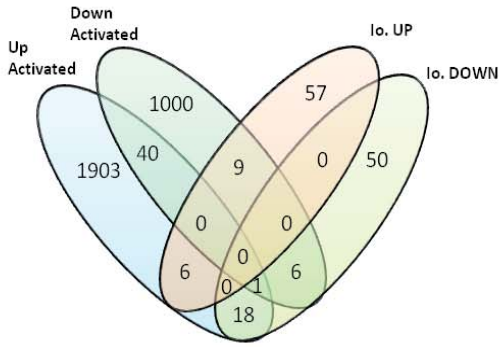
Common elements in "List 2" and "Io. UP":

CD160	DUSP2
B3GNT7	DUSP4

Common elements in "List 1" and "Io. UP":

PYCR1	TMEPAI
MOXD1	NMB
GNG4	

2.- Activated $CD56^{dim}CD16^{+}$ NK vs. $CD56^{dim}CD16^{+}$ NK cells



Common elements in "Up Activated" and "Io Down"

LTB	GZMH
GZMK	RNASE6
CTSW	IRF8
CD52	CCR5
MMP25	CCR1
GNLY	ITGAM
GZMA	LTC4S
XBP1	CD300A
HCST	C10orf128

Common elements in "Up Activated", "Down Activated" and "Io Down":
CCL3

Common elements in "Up Activated" and "Io. UP":

MAL	CYP1B1
FABP5	PYCR1
CD9	HSPB1

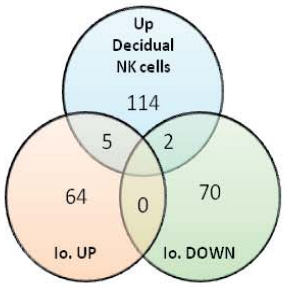
Common elements in "Down Activated" and "Io. DOWN":

NCAM1	PCSK5
GIMAP5	PLAC8
PTGDR	STOM

Common elements in "Down Activated" and "Io. UP":

HES6	RNF130
CD109	RAMP1
CD160	DUSP2
LAYN	PMEPA1
CTHRC1	

3.- Human Decidual NK cells



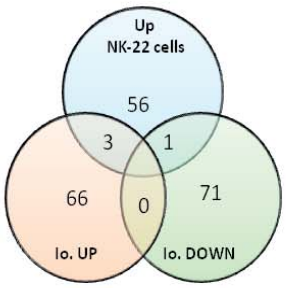
Common elements in "UP decidual NK cells" and "Io. UP":

CD9	up decidua vs CD56+bright and CD56+dim
ITGB5	up decidua vs CD56+bright and CD56+dim
PPAP2A	up decidua vs CD56+bright and CD56+dim
PLAU	up decidua vs CD56+bright and CD56+dim
LEF1	up decidua vs CD56+bright and CD56+dim

Common elements in "UP decidual NK cells" and "Io. DOWN":

SPRY2	up decidua vs CD56+ bright, up CD56+ bright vs CD56+ dim
GZMA	up decidua vs CD56+ bright, up CD56+ dim vs CD56+ bright

4.- Human NK-22 cells

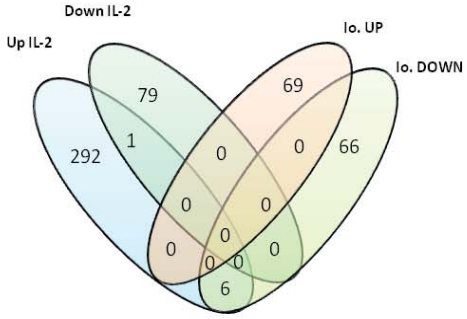


Common elements in "Up NK-22" and "Io Down"

KIAA1324	PPAP2A
LAYN	

Common elements in "Up NK-22" and "Io Down":
CXCR6

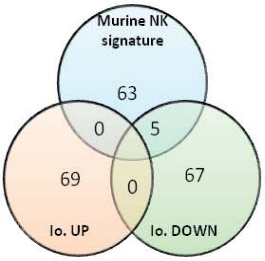
5.- Proteomics analysis of molecules enriched after stimulation of NK cells with IL-2



Common elements in "Up IL-2" and "lo. DOWN":

CTSW	PACSIN1
GIMAP5	CD300A
NCAM1	IFITM1

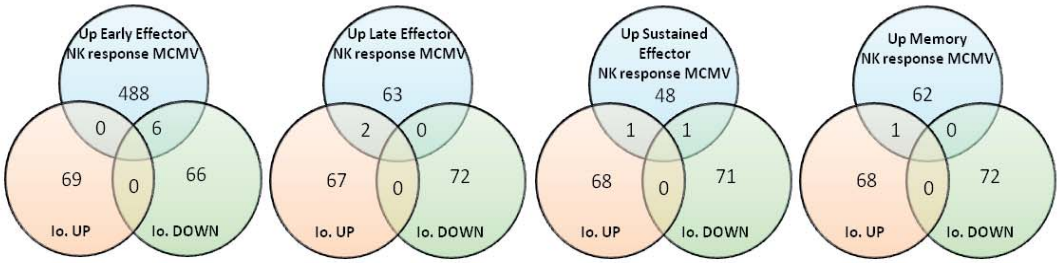
6.- Murine NK cells



Common elements in "Murine NK specific" and "lo. DOWN":

GZMA	ITGAM
STYK1	CTSW
SPRY2	

7.- Murine NK cells along MCMV infection



Common elements in "Early Eff" and "lo. DOWN":

CCL3
CCL4
CCR5
EGR1
EGR2
IRF8

Common elements in "Late eff" and "lo. UP":

CBFA2T3
CERCAM

Common elements in "Sustain Eff" and "lo. DOWN":

STOM

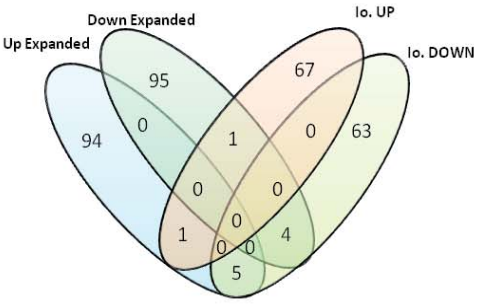
Common elements in "Sustain Eff" and "lo. UP":

B3GNT7

Common elements in "memory" and "lo. UP

HMOX1

8.- IL-2 expanded NK cells *vs.* freshly isolated human NK cells from metastatic patients



Common elements in "Up Expanded" and "lo. DOWN"

MMP25
LTB
NCR3
GZMK
GZMA

Common elements in "Down Expanded" and "lo. DOWN":

SELL
NCF2
MYOM2
TIMP1

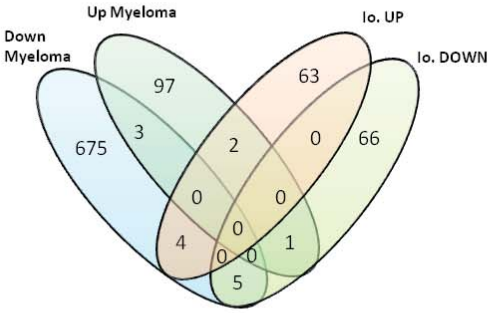
Common elements in "Down Expanded" and "lo. UP":

DUSP2

Common elements in "Up Expanded" and "lo. UP":

MAL

9.- NK cells from Myeloma patients vs. control samples



Common elements in "Down Myeloma" and "Io. DOWN":

ITGA6
CD300A
SPRY2
RNASE6
TNF

Common elements in "Down Myeloma" and "Io. UP":

IGJ
CD9
EPAS1
HSPB1

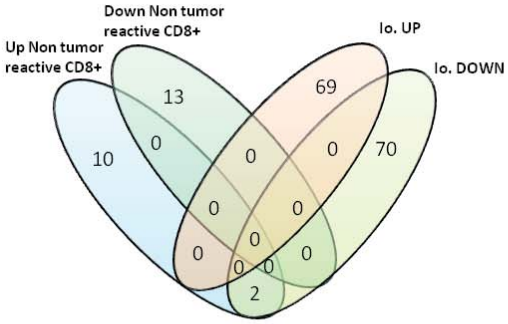
Common elements in "Up Myeloma" and "Io. DOWN":

C20orf197

Common elements in "Up Myeloma" and "Io. UP":

HPSE
CYP1B1

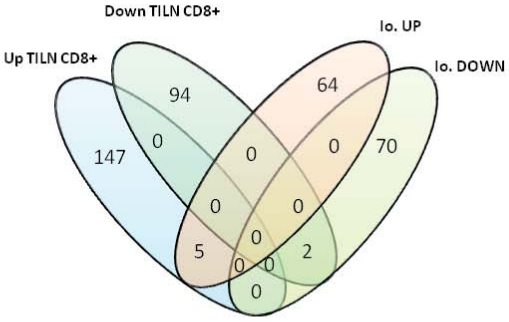
10.- Human non-tumour reactive CD8+ T cells *vs.* tumor reactive CD8+ T cells



Common elements in "Up Non tumor reactive CD8+" and "Io. DOWN":

EGR1
TIMP1

11.- Tumour specific human CD8+T cells infiltrating lymph nodes (TILN) *vs.* peripheral blood tumour infiltrating CD8+T cells



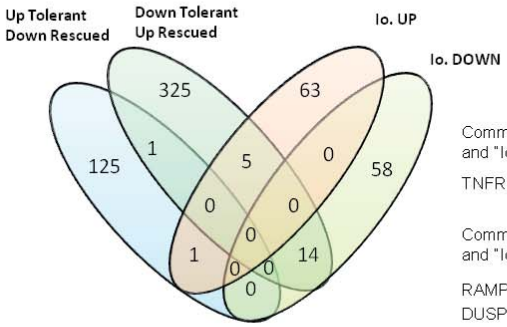
Common elements in "up TILN" and "Io. UP":

CD27
DUSP4
RDH10
SLC7A5
TNFRSF9

Common elements in "down TILN" and "Io. DOWN

CD300C
ITGAM

12.- Tolerant murine CD8⁺ T cells



Common elements in "Up Tolerant" and "Io. UP":

TNFRSF9

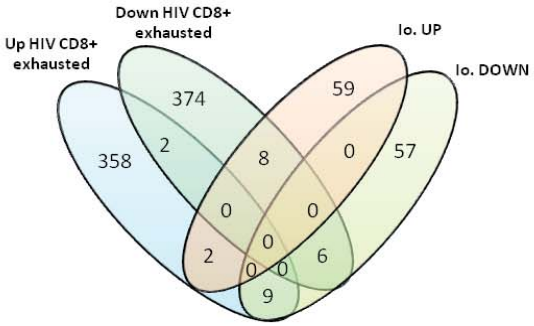
Common elements in "Up Rescued" and "Io. UP".:

RAMP1
DUSP2
VCAM1
LMNA
HMOX1

Common elements in "Up Rescued and "Io. DOWN":

MATK
EGR1
EGR2
TNF
GZMK
RNASE6
CCR5
IL18R1
CTSW
NT5E
PLAC8
CXCR6
HCST
CCL4

13.- Murine HIV exhausted CD8⁺ T cells



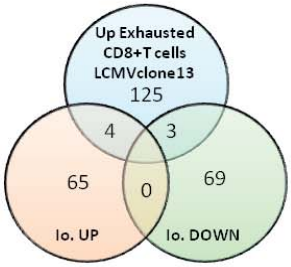
Common elements in "Up Exhausted" and "Io. UP":
TNFRSF9
IGJ

Common elements in "Up Exhausted" and "Io. DOWN":
IFITM1
STOM
CCL3
CCL3L3
GZMA
EGR1
CCR5
GZMH
CCR1

Common elements in "Down Exhausted" and "Io. DOWN":
TIMP1
IL18R1
ITGAM
TNF
SLAMF8
PCSK5

Common elements in "Down Exhausted" and "Io. UP":
CYP1B1
SLC7A5
MAL
LEF1
LMNA
CD9
NBL1
DUSP4

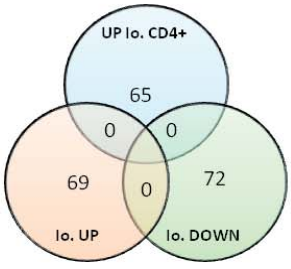
14.- Exhausted CD8⁺ T cells from mice chronically infected with LCMVclone13 *vs.* effector CD8⁺ T cells



Common elements in "Up Exhausted CD8+" and "Io. UP":
CD160
TNFRSF9
VCAM1
CD9

Common elements in "Up Exhausted CD8+" and "Io. DOWN
ITGB2
CCR2
EGR2

15.- Ionomycin induced anergic CD4⁺ T cells



" Education isn't something you can finish "

Issac Asimov

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